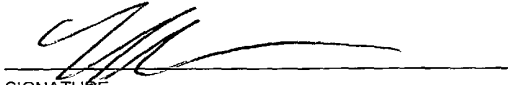



JC20 Rec'd PCT/PTO 29 MAR 2002

FORM PTO-1390 (Modified) (REV 5-93)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				053466-0325	
				U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5) Unassigned 10709501	
INTERNATIONAL APPLICATION NO. PCT/JP00/06802		INTERNATIONAL FILING DATE 29 September 2000		PRIORITY DATE CLAIMED 1 October 1999	
TITLE OF INVENTION PREVENTION AND TREATMENT OF BLOOD COAGULATION-RELATED DISEASES					
APPLICANT(S) FOR DO/EO/US Hiroyuki SAITO, Takehisa KITAZAWA, Kazutaka YOSHIHASHI, and Kunihiro HATTORI					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. 3. <input type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1). 4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19 th month from the earliest claimed priority date. 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). <input checked="" type="checkbox"/> has been transmitted by the International Bureau. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US) 6. <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)). 7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). <input type="checkbox"/> have been transmitted by the International Bureau. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. <input checked="" type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). 11. <input type="checkbox"/> Applicant claims small entity status under 37 CFR 1.27.					
Items 12. to 17. below concern other document(s) or information included:					
12. <input checked="" type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 13. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 14. <input checked="" type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 15. <input type="checkbox"/> A substitute specification. 16. <input type="checkbox"/> A change of power of attorney and/or address letter. 17. <input checked="" type="checkbox"/> Other items or information: Application Data Sheet (4 pages), Sequence Listing (57 pages), Receipt in the Case of an Original Deposit with Translation (2 pages)					

10087501 042202

JC13 Rec'd PCT/PTC 29 MAR 2002

U.S. APPLICATION NO. (If known, see 37 CFR 1.50) Unassigned 107089501		INTERNATIONAL APPLICATION NO. PCT/JP00/06802		ATTORNEY'S DOCKET NUMBER 053466-0325	
18. <input checked="" type="checkbox"/> The following fees are submitted:				CALCULATIONS PTO USE ONLY	
Basic National Fee (37 CFR 1.492(a)(1)-(5): Search Report has been prepared by the EPO or JPO.....\$890.00					
International preliminary examination fee paid to USPTO (37 CFR 1.482).....\$710.00					
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)).....\$740.00					
Neither international preliminary examination fee (37 CFR 1.482) nor International search fee (37 CFR 1.445(a)(2)) paid to USPTO.....\$1,040.00					
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....\$100.00					
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$890.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than 20 Months from the earliest claimed priority date (37 CFR 1.492(e))					
Claims	Number Filed	Included in Basic Fee	Extra Claims	Rate	
Total Claims	53	- 20	= 33	x \$18.00	\$594.00
Independent Claims	6	- 3	= 3	x \$84.00	\$252.00
Multiple dependent claim(s) (if applicable)				\$280.00	
TOTAL OF ABOVE CALCULATIONS =				\$1736.00	
Reduction by 1/2 for filing by small entity, if applicable.				\$0.00	
SUBTOTAL =				\$1736.00	
Processing fee of \$130.00 for furnishing English translation later the 20 months from the earliest claimed priority date (37 CFR 1.492(f)).				+	
TOTAL NATIONAL FEE =				\$1736.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +				+	
TOTAL FEES ENCLOSED =				\$1736.00	
				Amount to be: refunded \$	
				charged \$	
a. <input checked="" type="checkbox"/> A check in the amount of \$1736.00 to cover the above fees is enclosed.					
b. <input type="checkbox"/> Please charge my Deposit Account No. <u>19-0741</u> in the amount of \$0.00 to the above fees. A duplicate copy of this sheet is enclosed.					
c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>19-0741</u> . A duplicate copy of this sheet is enclosed.					
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.					
SEND ALL CORRESPONDENCE TO:					
Foley & Lardner Customer Number: 22428			SIGNATURE 		
			NAME HAROLD C. WEGNER		
22428			REGISTRATION NUMBER 25,258		
PATENT TRADEMARK OFFICE					

Application Data Sheet

Application Information

Application Type::	Regular
Subject Matter::	Utility
Suggested classification::	
Suggested Group Art Unit::	
CD-ROM or CD-R?::	None
Computer Readable Form (CRF)?::	Yes
Number of copies of CRF::	1
Title::	PREVENTION AND TREATMENT OF BLOOD COAGULATION-RELATED DISEASES
Attorney Docket Number::	053466-0325
Request for Early Publication?::	No
Request for Non-Publication?::	No
Suggested Drawing Figure::	1
Total Drawing Sheets::	14
Small Entity?::	No
Petition included?::	No
Secrecy Order in Parent Appl.?::	No

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Correspondence Information

Correspondence Customer Number:: 22428
E-Mail address:: hwegner@foleylaw.com

Representative Information

Representative Customer Number::	22428	
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Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This Application	National Stage of	PCT/JP00/06802	09/29/200

Foreign Priority Information

Country::	Application number::	Filing Date::	Priority Claimed::
Japan	11-281843	10/01/1999	Yes
Japan	11-282120	10/01/1999	Yes

Japan	11-282134	10/01/1999	Yes
Japan	11-282167	10/01/1999	Yes
Japan	11-282188	10/01/1999	Yes
Japan	11-282192	10/01/1999	Yes

Assignee Information

Assignee name::

CHUGAI SEIYAKU KABUSHIKI KAISHA

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Hiroyuki SAITO et al.

Title: PREVENTION AND TREATMENT
OF DISEASES ASSOCIATED
WITH BLOOD COAGULATION

Appl. No.: Unassigned

Filing Date: 03/29/2002

Examiner: Unassigned

Art Unit: Unassigned

PRELIMINARY AMENDMENT

Commissioner for Patents
Box PCT
Washington, D.C. 20231

Sir:

Prior to examination of the above-identified application, Applicants respectfully request that the above-identified application be amended as follows:

In the Claims:

In accordance with 37 C.F.R. §1.21, please substitute for claims 3, 5 – 8, 12 – 14, 16, 21 – 23, 25, 30 – 32, 34, 39 – 41, 43, 48 – 50, and 52 the following rewritten version of the same claims, as amended. The changes are shown explicitly in the attached "Marked-up Version of Amended Claims."

3. (Once Amended) The animal according to claim 1 wherein said animal cell is a mammalian cell.

5. (Once Amended) The animal according to claim 1 wherein said animal is a mouse.

6. (Once Amended) The animal according to claim 1 wherein said hypercoagulable state is indicated by at least one of the phenomena comprising an

increase in the plasma concentration of human tissue factor, a decrease in platelets, a decrease in fibrinogen, an increase in the concentration of soluble fibrin monomer complex, and an increase in the concentration of thrombin-antithrombin III complex.

7. (Once Amended) A method of generating the animal according to claim 1, wherein an animal cell to which the gene encoding human tissue factor (TF) or part thereof has been inserted and which is capable of expressing said gene is implanted to non-human animals and then an animal having a persistent hypercoagulable state is selected.

8. (Once Amended) A method of screening an anti-thrombotic agent which method comprises using the animal according to claim 1.

12. (Once Amended) The preventive or therapeutic agent according to claim 9 wherein said antibody is an altered antibody.

13. (Once Amended) The preventive or therapeutic agent according to claim 9 wherein said antibody is an altered antibody.

14. (Once Amended) The preventive or therapeutic agent according to claim 9 wherein said altered antibody is a chimereic antibody or a humanized antibody.

16. (Once Amended) The preventive or therapeutic agent according to claim 9 wherein said antibody is a modified antibody.

21. (Once Amended) The preventive or therapeutic agent according to claim 18 wherein said antibody is a recombinant antibody.

22. (Once Amended) The preventive or therapeutic agent according to claim 18 wherein said antibody is an altered antibody.

23. (Once Amended) The preventive or therapeutic agent according to claim 18 wherein said antibody is an altered antibody.

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~~29.~~ (Once Amended) The preventive or therapeutic agent according to claim 18 wherein said altered antibody is a chimereic antibody or a humanized antibody.

25. (Once Amended) The preventive or therapeutic agent according to claim 18 wherein said antibody is a modified antibody.

30. (Once Amended) The preventive or therapeutic agent according to claim 27 wherein said antibody is a recombinant antibody.

31. (Once Amended) The preventive or therapeutic agent according to claim 27 wherein said antibody is an altered antibody.

32. (Once Amended) The preventive or therapeutic agent according to claim 27 wherein said altered antibody is a chimeric antibody or a humanized antibody.

34. (Once Amended) The preventive or therapeutic agent according to claim 27 wherein said antibody is a modified antibody.

39. (Once Amended) The preventive or therapeutic agent according to claim 36 wherein said antibody is a recombinant antibody.

40. (Once Amended) The preventive or therapeutic agent according to claim 36 wherein said antibody is an altered antibody.

41. (Once Amended) The preventive or therapeutic agent according to claim 36 wherein said altered antibody is a chimeric antibody or a humanized antibody.

43. (Once Amended) The preventive or therapeutic agent according to claim 36 wherein said antibody is a modified antibody.

48. The preventive or therapeutic agent according to claim 45 wherein said antibody is a recombinant antibody.

52. (Once Amended) The preventive or therapeutic agent according to claim 43 wherein said antibody is a modified antibody.

Applicants respectfully request that the foregoing amendments be made prior to examination of the present application. The amendments are made to correct multiple dependencies and do not change the scope of the invention.

Harold C. Wegner
Attorney for Applicants
Registration No. 25,258

MARKED UP VERSION OF AMENDED CLAIMS

3. (Once Amended) The animal according to claim 1 [or 2] wherein said animal cell is a mammalian cell.

5. (Once Amended) The animal according to [any one of claims 1 to 4] claim 1 wherein said animal is a mouse.

6. (Once Amended) The animal according to [any one of claims 1 to 5] claim 1 wherein said hypercoagulable state is indicated by at least one of the phenomena comprising an increase in the plasma concentration of human tissue factor, a decrease in platelets, a decrease in fibrinogen, an increase in the concentration of soluble fibrin monomer complex, and an increase in the concentration of thrombin-antithrombin III complex.

7. (Once Amended) A method of generating the animal according to [any one of claims 1 to 6] claim 1, wherein an animal cell to which the gene encoding human tissue factor (TF) or part thereof has been inserted and which is capable of expressing said gene is implanted to non-human animals and then an animal having a persistent hypercoagulable state is selected.

8. (Once Amended) A method of screening an anti-thrombotic agent which method comprises using the animal according to [any one of claims 1 to 6] claim 1.

12. (Once Amended) The preventive or therapeutic agent according to claim 9 [or 11] wherein said antibody is an altered antibody.

13. (Once Amended) The preventive or therapeutic agent according to claim 9 [or 12] wherein said antibody is an altered antibody.

Atty. Dkt. No. 053466-0325

14. (Once Amended) The preventive or therapeutic agent according to claim 9 [,12, or 13] wherein said altered antibody is a chimereic antibody or a humanized antibody.

16. (Once Amended) The preventive or therapeutic agent according to claim 9 [or any one of claims 12-15] wherein said antibody is a modified antibody.

21. (Once Amended) The preventive or therapeutic agent according to claim 18 [or 20] wherein said antibody is a recombinant antibody.

22. (Once Amended) The preventive or therapeutic agent according to claim 18 [or 21] wherein said antibody is an altered antibody.

23. (Once Amended) The preventive or therapeutic agent according to claim 18 [, 21, or 22] wherein said altered antibody is a chimereic antibody or a humanized antibody.

25. (Once Amended) The preventive or therapeutic agent according to claim 18 [or any one of claims 21-24] wherein said antibody is a modified antibody.

30. (Once Amended) The preventive or therapeutic agent according to claim 27 [or 29] wherein said antibody is a recombinant antibody.

31. (Once Amended) The preventive or therapeutic agent according to claim 27 [or 30] wherein said antibody is an altered antibody.

32. (Once Amended) The preventive or therapeutic agent according to claim 27 [, 30, or 31] wherein said altered antibody is a chimeric antibody or a humanized antibody.

34. (Once Amended) The preventive or therapeutic agent according to claim 27 [or any one of claims 30-33] wherein said antibody is a modified antibody.

39. (Once Amended) The preventive or therapeutic agent according to claim 36 [or 38] wherein said antibody is a recombinant antibody.

40. (Once Amended) The preventive or therapeutic agent according to claim 36 [or 39] wherein said antibody is an altered antibody.

41. (Once Amended) The preventive or therapeutic agent according to claim 36 [, 39, or 40] wherein said altered antibody is a chimeric antibody or a humanized antibody.

43. (Once Amended) The preventive or therapeutic agent according to claim 36 [or any one of claims 39-42] wherein said antibody is a modified antibody.

48. The preventive or therapeutic agent according to claim 45 [or 47] wherein said antibody is a recombinant antibody.

49. (Once Amended) The preventive or therapeutic agent according to claim 45 [or 48] wherein said antibody is an altered antibody.

50. (Once Amended) The preventive or therapeutic agent according to claim 45 [, 48, or 49] wherein said altered antibody is a chimeric antibody or a humanized antibody.

52. (Once Amended) The preventive or therapeutic agent according to claim 43 [or any one of claims 48-51] wherein said antibody is a modified antibody.

DESCRIPTION

PREVENTION AND TREATMENT OF BLOOD COAGULATION-RELATED
DISEASES

5

FIELD OF THE INVENTION

The present invention relates to a method of generating an animal model having a persistently hypercoagulable state, preventive or therapeutic agents for diseases having a persistent hypercoagulable state, preventive or therapeutic agents for a hypercoagulable state resulting from infections, preventive or therapeutic agents for venous or arterial thrombosis, and preventive or therapeutic agents for diseases resulting from the hypertrophy of vascular media.

BACKGROUND ART

Blood coagulation is a reaction in which serine protease precursors are successively activated by activated-form proteases, which finally generate thrombin thereby leading to fibrin formation. Thrombosis arises as a consequence of an excessively enhanced blood coagulation reaction that was caused by changes in the plasma coagulation and fibrinolytic system, and in the functions of platelets, leucocytes and vascular endothelial cells associated with the progression of various disease states. The initiating factor of the blood coagulation reaction is tissue factor. In acute coronary syndromes such as acute myocardial infarction and unstable angina, the blood coagulation reaction is triggered when tissue factor occurring in abundance in the plaques formed after the progression of arterial sclerosis is exposed to the blood due to the rhexis of plaques.

In the disseminated intravascular coagulation syndrome associated with sepsis and malignant tumors, activated monocytes and macrophages express tissue factor

- 2 -

or tumor cells express tissue factor thereby causing enhanced blood coagulation. Once tissue factor comes into contact with the blood, the blood coagulation reaction proceeds in a very short period of time and leads to the formation of blood clots. Thus, in order to prevent thrombus formation, it is necessary to block blood coagulation reactions that may be triggered at any time or that may be constantly occurring. Therefore, an experimental model that exhibits a hypercoagulable state on a persistent basis is essential for the development of effective anti-thrombotic agents. In any of the conventionally known thrombotic models, thrombus formation is induced in a short period of time.

Thus, according to one aspect of the present invention, there is provided an experimental model in which a hypercoagulable state persists by bringing human tissue factor into contact with the blood on a persistent basis.

Blood coagulation is a reaction in which serine protease precursors are successively activated by activated-form proteases, which finally generate thrombin thereby leading to fibrin formation. Thrombosis arises as a consequence of an excessively enhanced blood coagulation reaction that was caused by changes in the plasma coagulation and fibrinolytic system, and in the functions of platelets, leucocytes and vascular endothelial cells associated with the progression of various disease states. The initiating factor of the blood coagulation reaction is tissue factor (TF).

In acute coronary syndromes such as acute myocardial infarction and unstable angina, the blood coagulation reaction is triggered when tissue factor occurring in abundance in the plaques formed after the progression of arterial sclerosis is exposed to the blood due to the rupture of plaques. In disseminated intravascular coagulation syndrome associated with sepsis and malignant tumors, activated monocytes and macrophages express TF or

the arteriosclerotic plaques and the ensuing thrombus formation is an important factor in the onset mechanism of the disease.

5 It has also been demonstrated that tissue factor (TF), an initiating factor for thrombus formation, is being excessively expressed on the cell surface and the extracellular interstitium in the plaque, and thus it is believed that the exposure of tissue factor (TF) to the blood resulting from the rupture of plaques is a major
10 factor for thrombus formation.

Thus, there is a great need for the development of a novel drug for preventing or treating arterial thrombosis.

15 Thus, according to the fifth aspect of the present invention, there is provided a novel preventive or therapeutic agent for arterial thrombosis.

Percutaneous transluminal coronary angioplasty (PTCA) occupies an important position in the treatment of coronary heart diseases. But restenosis that occurs
20 several months after the operation hinders the effectiveness of the treating method and thus is posing a problem. As a cause of restenosis, it is becoming increasingly clear, thrombus formation during the acute phase and the subacute phase resulting from the injuries to endothelial cells is important. The contact with the
25 blood of tissue factor (TF) expressed by the injured endothelial cells and the smooth muscles and fibroblasts in the subendothelial tissue is important for thrombus formation. The cells in the blood vessel wall grow so as
30 to cover the resulting thrombi and thereby narrow the area of the lumen in the blood vessel. The growth of the blood vessel tissue per se and the constriction of the blood vessel diameter also contribute to the narrowing of the area of the lumen in the blood vessel, and they
35 provide a direct factor for restenosis. Thus, there is a great need for a novel drug that can prevent or treat restenosis.

Thus, according to the sixth aspect of the present invention, there is provided a novel preventive or therapeutic agent for diseases caused by the hypertrophy of vascular media.

5

DISCLOSURE OF THE INVENTION

After intensive and extensive research to resolve the above first problem, the inventors of the present invention have found out that by implanting, into an experimental animal, an animal cell capable of constantly expressing human tissue factor by introducing therein the gene of a human tissue factor (TF) and thereby increasing the concentration of human tissue factor in the animal, the hypercoagulable state of said animal can be maintained for a long period of time, and thereby have completed the present invention.

Thus, according to the first aspect, the present invention provides an experimental animal having implanted therein an animal cell to which the gene encoding human tissue factor (TF) or part thereof has been inserted and which is capable of expressing said gene, said animal being a non-human animal in which a hypercoagulable state persists for a long period of time.

The part of said human tissue factor is for example a human tissue factor that lacks the intracellular region. Said animal cell is preferably a mammalian cell. Said mammalian cell is preferably a human myeloma cell. Said animal is preferably a mouse. Said hypercoagulable state is indicated by at least one of the phenomena comprising an increase in the plasma concentration of human tissue factor, a decrease in platelets, a decrease in fibrinogen, an increase in the concentration of soluble fibrin monomer complex, and an increase in the concentration of thrombin-antithrombin III complex.

The present invention also provides a method of generating the above animal, wherein an animal cell to which the gene encoding human tissue factor (TF) or part

a hypercoagulable state resulting from infections, said agent comprising an antibody against human tissue factor (human TF).

5 The above antibody is for example a polyclonal antibody. The above antibody is preferably a monoclonal antibody. The above antibody is preferably a recombinant antibody. The above antibody is preferably an altered antibody. The above altered antibody is preferably a chimeric antibody or a humanized antibody. The above
10 humanized antibody is a humanized antibody of version b-b, i-b, or i-b2. The above antibody is for example an antibody modification. The above modified antibody is for example an antibody fragment Fab, F(ab')₂, or Fv, or a single chain Fv (scFv).

15 After intensive and extensive research to resolve the above fourth problem, the inventors of the present invention have found out that an antibody (anti-human TF antibody, or sometimes referred to as anti-TF antibody) against human tissue factor can prevent or treat venous
20 thrombosis.

 Thus, according to the fourth aspect, the present invention provides a preventive or therapeutic agent for venous thrombosis, said agent comprising an antibody against human tissue factor (human TF).

25 The above antibody is for example a polyclonal antibody. The above antibody is preferably a monoclonal antibody. The above antibody is preferably a recombinant antibody. The above antibody is preferably an altered antibody. The above altered antibody is preferably a
30 chimeric antibody or a humanized antibody. The above humanized antibody is a humanized antibody of version b-b, i-b, or i-b2. The above antibody is for example an antibody modification. The above antibody modification is for example an antibody fragment Fab, F(ab')₂, or Fv, or a single chain Fv (scFv).
35

 After intensive and extensive research to resolve the above fifth problem, the inventors of the present

invention have found out that an antibody (anti-human TF antibody, or sometimes referred to as anti-TF antibody) against human tissue factor can prevent or treat arterial thrombosis.

5 Thus, according to the fifth aspect, the present invention provides a preventive or therapeutic agent for arterial thrombosis, said agent comprising an antibody against human tissue factor (human TF).

10 The above antibody is for example a polyclonal antibody. The above antibody is preferably a monoclonal antibody. The above antibody is preferably a recombinant antibody. The above antibody is preferably an altered antibody. The above altered antibody is preferably a chimeric antibody or a humanized antibody. The above
15 humanized antibody is a humanized antibody of version b-b, i-b, or i-b2. The above antibody is for example an antibody modification. The above antibody modification is for example an antibody fragment Fab, F(ab')₂, or Fv, or a single chain Fv (scFv).

20 After intensive and extensive research to resolve the above sixth problem, the inventors of the present invention have found out that an antibody (anti-human TF antibody, or sometimes referred to as anti-TF antibody) against human tissue factor can prevent or treat diseases
25 caused by the hypertrophy of vascular media.

 Thus, according to the sixth aspect, the present invention provides a preventive or therapeutic agent for diseases caused by the hypertrophy of vascular media, said agent comprising an antibody against human tissue
30 factor (human TF).

 The above antibody is for example a polyclonal antibody. The above antibody is preferably a monoclonal antibody. The above antibody is preferably a recombinant antibody. The above antibody is preferably an altered
35 antibody. The above altered antibody is preferably a chimeric antibody or a humanized antibody. The above humanized antibody is a humanized antibody of version b-

b, i-b, or i-b2. The above antibody is for example an antibody modification. The above antibody modification is for example an antibody fragment Fab, F(ab')₂, or Fv, or a single chain Fv (scFv).

5

BRIEF EXPLANATION OF THE DRAWINGS

Figure 1 is a graph that compares the antigen binding activity of a H chain chimeric/L chain chimeric antibody, a H chain humanized version b/L chain humanized version b antibody, a H chain humanized version i/L chain humanized version b antibody, and a H chain humanized version i/L chain humanized version b2 antibody.

Figure 2 is a graph that compares the activity of neutralizing human TF (the activity of TF to inhibit the production of Factor Xa) of a H chain chimeric/L chain chimeric antibody, a H chain humanized version b/L chain humanized version b antibody, a H chain humanized version i/L chain humanized version b antibody, and a H chain humanized version i/L chain humanized version b2 antibody.

Figure 3 is a graph that compares the activity of neutralizing human TF (the activity of TF to inhibit the production of Factor X) of a H chain chimeric/L chain chimeric antibody, a H chain humanized version b/L chain humanized version b antibody, a H chain humanized version i/L chain humanized version b antibody, and a H chain humanized version i/L chain humanized version b2 antibody.

Figure 4 is a graph that compares the activity of neutralizing human TF (the activity of TF to inhibit plasma coagulation) of a H chain chimeric/L chain chimeric antibody, a H chain humanized version b/L chain humanized version b antibody, a H chain humanized version i/L chain humanized version b antibody, and a H chain humanized version i/L chain humanized version b2 antibody.

Figure 5 is a graph showing changes in tumor volume

with time after the implantation of tumor cells in the mice implanted with the cells to which gene of human tissue factor has been introduced (dotted line) and in the mice implanted with the cells to which said gene has not been introduced (solid line).

Figure 6 is a graph showing changes in the plasma concentration of human tissue factor with time after the implantation of tumor cells in the mice implanted with the cells to which gene of human tissue factor has been introduced (dotted line) and in the mice implanted with the cells to which said gene has not been introduced (solid line).

Figure 7 is a graph showing changes in platelet counts with time after the implantation of tumor cells in the mice implanted with the cells to which gene of human tissue factor has been introduced (dotted line) and in the mice implanted with the cells to which said gene has not been introduced (solid line).

Figure 8 is a graph showing changes in the plasma concentration of fibrinogen with time after the implantation of tumor cells in the mice implanted with the cells to which gene of human tissue factor has been introduced (dotted line) and in the mice implanted with the cells to which said gene has not been introduced (solid line). The points indicate relative values, in which the concentration of fibrinogen in the control mice to which tumor cells have not been implanted (normal) is expressed as 100%.

Figure 9 is a graph showing changes in the plasma concentration of soluble fibrin monomer complex (SFMC) with time after the implantation of tumor cells in the mice implanted with the cells to which gene of human tissue factor has been introduced (dotted line) and in the mice implanted with the cells to which said gene has not been introduced (solid line).

Figure 10 is a graph showing changes in the plasma concentration of thrombin-antithrombin III complex (TAT)

acid sequence encoded thereby are also known (H. Morrissey et al., Cell, Vol. 50, p. 129-135 (1987)). The base sequence encoding the full-length human tissue factor and the corresponding amino acid sequence are set forth in SEQ ID NO: 103 and 104. According to the present invention, there may be used a gene encoding TF from which the intracellular region has been removed or a gene encoding the portion that retains the activity of initiating the blood coagulation system.

As a vector for introducing this gene into an animal cell and expressing it, any expression vector that functions in animal cells can be used, including, for example, pCOS1, pSV2-neo, pMAM-neo, and pSG5. In accordance with the present invention, a commonly used useful promoter, the human tissue factor gene, and a poly A signal, to 3'-end downstream thereof, can be functionally linked and can be expressed. As the promoter/enhancer, there can be mentioned human cytomegalovirus immediate early promoter/enhancer, viral promoters such as promoters of retrovirus, polyoma virus, adenovirus, and simian virus 40 (SV40), and promoters/enhancers derived from mammalian cells such as human elongation factor 1 α (HEF1 α). For expression vectors, as the replicator, there can be used those derived from SV40, polyoma virus, adenovirus and the like. Furthermore, for the expression vector can be contained as selectable markers the phosphotransferase APH (3') II or I (neo) gene, the thymidine kinase (TK) gene, the dihydrofolate reductase (dhfr) gene and the like.

As the method of introducing a gene into a cell, there can be used the electroporation method, the calcium phosphate method, the lipofection method and the like. As the cell for introducing the expression vector, any cell can be used as long as it can be grafted to an animal cell. For this purpose, various cultured cells may be used, including for example a mammalian cell such

therapeutic effect on the persistence of a hypercoagulable state due to TF, monoclonal antibody is preferable. In addition, chimeric antibody, humanized antibody or single chain Fv and so forth based on
5 monoclonal antibody can also be used, while humanized antibody is particularly preferable.

Although the antibody used in the fifth aspect of the present invention may be either polyclonal antibody or monoclonal antibody provided it has a preventive or
10 therapeutic effect on the persistence of a hypercoagulable state due to TF, monoclonal antibody is preferable. In addition, chimeric antibody, humanized antibody or single chain Fv and so forth based on monoclonal antibody can also be used, while humanized
15 antibody is particularly preferable.

Although the antibody used in the sixth aspect of the present invention may be either polyclonal antibody or monoclonal antibody provided it has a preventive or
20 therapeutic effect on the persistence of a hypercoagulable state due to TF, monoclonal antibody is preferably. In addition, chimeric antibody, humanized antibody or single chain Fv and so forth based on monoclonal antibody can also be used, while humanized antibody is particularly preferable.

25 1. Anti-human TF antibody

The anti-human TF antibody used in the present invention may be of any origin, type (monoclonal or polyclonal) and form provided it has the effect of preventing or treating viral hemorrhagic fever.

30 The anti-human TF antibody used in the present invention can be obtained as polyclonal or monoclonal antibody using a known means. Monoclonal antibody of mammalian origin is particularly preferable as the anti-human TF antibody used in the present invention.
35 Monoclonal antibody of mammalian origin includes that produced in hybridomas as well as that produced in a host transformed with an expression vector containing antibody

gene by genetic engineering techniques. This antibody is an antibody that inhibits the induction of thrombus by human TF by binding with human TF.

2. Antibody-Producing Hybridoma

5 Monoclonal antibody-producing hybridoma can basically be produced in the following manner using known technology. Namely, using human TF or a portion (fragment) of it as sensitizing antigen, this is immunized in accordance with ordinary immunization
10 methods, the resulting immunocytes are fused with known parent cells in accordance with ordinary cell fusion methods, and those cells that produce monoclonal antibody are screened in accordance with ordinary screening methods to produce monoclonal antibody.

15 More specifically, monoclonal antibody should be produced in the manner described below.

To begin with, human TF used as sensitizing antigen for antibody acquisition is obtained by expressing the TF gene/amino acid sequence disclosed in J.H. Morissey, et
20 al., Cell, Vol. 50, p. 129-135 (1987). Namely, gene sequence coding for human TF is inserted into a known expression vector to transform suitable host cells followed by purifying the target human TF protein present in the host cells or culture supernatant using a known
25 method. This method is described in Reference Example 1 of the present specification. Moreover, the human TF used as antigen can be used by extracting and purifying from a TF-containing biological material such as human placenta according to the method described in Reference
30 Example 2.

Next, this purified human TF protein is used as sensitizing antigen. Alternatively, soluble TF from which the membrane permeating region of the C-terminal of human TF has been removed can be produced by, for
35 example, genetic recombination, and this can also be used as sensitizing antigen.

Although there are no particular restrictions on the

mammal that is sensitized with sensitizing antigen, it is preferable to select a mammal in consideration of compatibility with the parent cells used in cell fusion, typical examples of which include rodents such as mice, rats, hamsters, or rabbits and monkeys.

Immunization of animals with sensitizing antigen is performed in accordance with known methods. For example, as a typical immunization method, immunization is performed by injecting sensitizing antigen into the abdominal cavity or under the skin of the mammal. More specifically, sensitizing antigen is diluted to a suitable volume with phosphate-buffered saline (PBS) or physiological saline, and the resulting suspension is mixed with a suitable amount of ordinary adjuvant such as Freund's complete adjuvant as desired followed by emulsifying and administering in multiple doses to mammals every 4-21 days. In addition, a suitable carrier can also be used when immunizing with sensitizing antigen.

After immunizing the mammals in this manner and confirming that antibody has risen to the desired level in the serum, immunocytes are sampled from the mammals and applied to cell fusion. However, spleen cells are a particularly preferable example of immunocytes.

Mammalian myeloma cells are used for the other parent cells fused with the above immunocytes. Various known cell lines are used for these myeloma cells, preferable examples of which include P3 (P3x63Ag8.653) (Kearney, J.F. et al., J. Immunol. (1979) 123, 1548-1550), P3x63Ag8U.1 (Yelton, D.E. et al., Current Topics in Microbiology and Immunology (1978) 81, 1-7), NS-1 (Kohler, G. and Milstein, C., Eur. J. Immunol. (1976) 6, 511-519), MPC-11 (Margulies, D.H. et al., Cell (1976) 8, 405-415), SP2/0 (Shulman, M. et al., Nature (1978) 276, 269-270), F0 (de St. Groth, S.F. and Scheidegger, D.J., J. Immunol. Methods (1980) 35, 1-21), S194 (Trowbridge, I.S., J. Exp. Med. (1978) 148, 313-323) and R210 (Galfre,

G. et al., Nature (1979) 277, 131-133).

Cell fusion of the above immunocytes and myeloma cells can basically be carried out in compliance with known methods such as the method of Milstein, et al.

5 (Galfre G. and Milstein, C., Methods Enzymol. (1981) 73, 3-46).

More specifically, the above cell fusion is carried out, for example, in ordinary nutrient culture media in the presence of cell fusion promoter. Examples of cell
10 fusion promoters used include polyethylene glycol (PEG) and Sendai virus (HVJ). Moreover, an assistant such as dimethylsulfoxide can be added to further enhance fusion efficiency as desired.

The usage ratio of immunocytes and myeloma cells can
15 be set arbitrarily. For example, the number of immunocytes is preferably 1-10 times the number of myeloma cells. Examples of culture media used in the above cell fusion include RPMI1640 culture medium, MEM culture medium and other ordinary culture media used in
20 this type of cell culturing that is suitable for growth of the above myeloma cell lines. Moreover, serum supplement such as fetal calf serum (FCS) can also be used in combination with the above media.

Cell fusion is carried out by adequately mixing
25 prescribed amounts of the above immunocytes and myeloma cells in the above culture media, adding PEG solution (for example, that having a molecular weight of about 1000-6000) warmed in advance to about 37°C at a concentration of usually 30-60% (w/v) and mixing to form
30 the target fused cells (hybridoma). Subsequently, a suitable amount of culture media is sequentially added, and cell fusion agents and so forth undesirable for hybridoma growth are removed by repeated removal of supernatant by centrifugation.

35 The hybridoma obtained in this manner is selected by culturing in an ordinary selective culture medium such as HAT culture medium (culture medium containing

hypoxanthine, aminopterin and thymidine). Culturing in the above HAT culture medium is continued for an adequate amount of time (normally from several days to several weeks) for killing cells other than the target hybridoma cells (non-fused cells). Next, routine critical dilution is performed followed by screening for hybridoma that produces the target antibody and monocloning.

In addition, besides obtaining the above hybridoma by immunizing animals other than humans with antigen, a desired human antibody having binding activity to human TF can be obtained by sensitizing human lymphocytes to human TF in vitro, and fusing the sensitized lymphocytes with human myeloma cells such as myeloma cell line U266 having permanent mitotic ability (refer to Japanese Examined Patent Publication No. 1-59878). Moreover, human antibody to anti-human TF may also be acquired from attenuated cells by administering human TF serving as antigen to transgenic animals having all or a portion of the human antibody gene repertoire, acquiring anti-human TF antibody-producing cells and attenuating those cells (refer to International Unexamined Patent Application No. WO 94/25585, WO 93/12227, WO 92/03918, WO 94/02602, WO 96/34096 and WO 96/33735).

Hybridoma that produces monoclonal antibody obtained in this manner can be sub-cultured in ordinary culture media, and can be stored for a long period of time in liquid nitrogen.

In order to acquire monoclonal antibody from said hybridoma, said hybridoma is cultured in accordance with routine methods followed by obtaining the culture supernatant, or the hybridoma can be administered to a compatible mammal to proliferate in that mammal followed by obtaining in the form of the ascites. The former method is suitable for obtaining highly pure antibody, while the latter method is suitable for large volume production of antibody.

An example of monoclonal antibody production is

specifically described in Reference Example 2. In this example, six types of monoclonal antibodies referred to as ATR-2, 3, 4, 5, 7 and 8 are obtained. Although all of these can be used in the present invention, ATR-5 is particularly preferable.

3. Recombinant Antibody

In the present invention, recombinant antibody produced using genetic recombination technology by cloning antibody gene from hybridoma, incorporating in a suitable vector and introducing this into a host can be used as monoclonal antibody (refer to, for example, Vandamme, A.M. et al., Eur. J. Biochem. (1990) 192, 767-775).

More specifically, mRNA that codes for the variable region (V) of anti-human TF antibody is isolated from hybridoma that produces anti-human TF antibody. Isolation of mRNA is carried out by a known method such as guanidine ultracentrifugation (Chirgwin, J.M. et al., Biochemistry (1979) 18, 5294-5299) or the AGPC method (Chomczynski, P. and Sacchi, N., Anal. Biochem. (1987) 162, 156-159) to prepare total RNA, followed by preparation of the target mRNA using an mRNA Purification Kit (Pharmacia). In addition, mRNA can also be prepared directly by using the QuickPrep mRNA Purification Kit (Pharmacia).

cDNA of the antibody V region is synthesized from the resulting mRNA using reverse transcriptase. Synthesis of cDNA is carried out using the AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Seikagaku Co.). In addition, synthesis and amplification of cDNA can also be carried out by using the 5'-Ampli FINDER RACE Kit (Clontech) and the 5'-RACE method using PCR (Frohman, M.A. et al., Proc. Natl. Acad. Sci. USA (1988) 85, 8998-9002, Belyavsky, A. et al., Nucleic Acids Res. (1989) 17, 2919-2932).

The target DNA fragment is purified from the resulting PCR product and linked with vector DNA.

increase the amount of breast milk containing the desired antibody produced by that transgenic goat (Ebert, K.M. et al., Bio/Technology (1994) 12, 699-702).

5 An example of a production method of recombinant antibody is specifically described in Reference Example 3.

4. Altered Antibody

10 In the present invention, in addition to the above-mentioned antibodies, genetic recombinant antibody that has been artificially altered for the purpose of decreasing heterogenic antigenicity with respect to humans can also be used, examples of which include chimeric antibody and humanized antibody. These altered antibodies can be produced using known methods.

15 Chimeric antibody is obtained by linking DNA that codes for the antibody V region in the manner described above and DNA that codes for human antibody C region, incorporating this in an expression vector and introducing into a host to produce antibody. Chimeric antibody that is useful in the present invention can be obtained using this known method.

20 Humanized antibody is also referred to as reshaped human antibody. This is the result of transplanting the complementarity determining region (CDR) of antibody of a mammal other than a human, such as mouse antibody, into the complementarity determining region of human antibody, and typical genetic recombination techniques are known for this (refer to European Unexamined Patent Publication No. EP 125023 and WO 96/02576).

30 More specifically, a DNA sequence designed so as to link the CDR of mouse antibody with the framework region (FR) of human antibody is synthesized by PCR using as primer a plurality of oligonucleotides prepared so as to have a portion that overlaps the terminal regions of both CDR and FR (refer to the method described in the publication of WO 98/13388).

A region in which the complementarity determining

region forms a satisfactory antigen binding site is selected for the framework region of the human antibody that is linked by way of CDR. The amino acids of the framework region in the variable region of the antibody
5 may be substituted as necessary so that the complementarity determining region of reshaped human antibody forms an appropriate antigen binding site (Sato, K. et al., Cancer Res. (1993) 53, 851-856).

The C region of human antibody is used for the C
10 region of chimeric antibody and humanized antibody, and for example, Cy1, Cy2, Cy3 and Cy4 can be used in the H chain, while Ck and Cl can be used in the L chain. In addition, human antibody C region may be modified to improve the stability of the antibody or its production.

15 Chimeric antibody is composed of the variable region of antibody originating in a mammal other than humans and the constant region of human antibody. On the other hand, humanized antibody is composed of the complementarity determining region of an antibody
20 originating in a mammal other than humans and the framework region and C region of human antibody. Since humanized antibody has decreased antigenicity in the human body, it is useful as an effective ingredient of the therapeutic agent of the present invention.

25 The production method of chimeric antibody is specifically described in Reference Example 4.

In addition, the production method of humanized antibody is specifically described in Reference Example
5. In this reference example, versions a, b, c, d, e, f,
30 g, h, i, j, b1, d1, b3 and d3 having the amino acid sequences shown in Tables 1 and 2 were used as the humanized heavy chain (H chain) variable region (V region).

5. Modified Antibody Substances

The antibody used in the present invention may be an antibody fragment or modified antibody substance provided it binds to human TF and inhibits human TF activity. For example, examples of antibody fragments include single chain Fv (scFv) in which Fab, F(ab')₂, Fv or H chain or L chain Fv is linked with a suitable linker.

More specifically, either antibody is treated with an enzyme such as papain or pepsin to produce antibody fragments, or a gene is constructed that codes for these antibody fragments, after which a fragment is inserted into an expression vector and expressed in a suitable host (refer to, for example, Co, M.S. et al., J. Immunol. (1994) 152, 2968-2976, Better, M. & Horowitz, A.H., Methods in Enzymology (1989) 178, 476-496, Plueckthun, A. & Skerra, A., Methods in Enzymology (1989) 178, 497-515, Lamoyi, E., Methods in Enzymology (1986) 121, 652-663, Rousseaux, J. et al., Methods in Enzymology (1986) 121, 663-669, and Bird, R.E. et al., TIBTECH (1991) 9, 132-137).

scFv is obtained by linking antibody H chain V region and L chain V region. In this scFv, the H chain V region and L chain V region are linked by means of a linker, and preferably by means of a peptide linker (Huston, J.S. et al., Proc. Natl. Acad. Sci., USA (1988) 85, 5879-5883). The H chain V region and L chain V region in scFv may be of any origin described as antibody in the present specification. An arbitrary single chain peptide comprised of, for example, 12-19 amino acid residues is used for the peptide linker that links the V regions.

DNA that codes for scFv is obtained by using as template the portion of DNA coding for H chain or H chain V region and DNA coding for L chain or L chain V region of the above antibody that codes for the entire or desired amino acid sequence of those sequences, amplifying by PCR using a primer pair that defines both

retrovirus, poliovirus, adenovirus, and simean virus 40 (SV40), as well as promoter/enhancer originating in mammalian cells such as human elongation factor 1 α (HEF1 α).

5 Gene expression can be carried out easily according to the method of Mulligan, et al. (Nature (1979) 277, 108-114) in the case of using SV40 promoter/enhancer, or according to the method of Mizushima, et al. (Nucleic Acid Res. (1990) 18, 5322) in the case of using HEF1 α
10 promoter/enhancer.

 In the case of E. coli, said gene can be expressed by functionally coupling a commonly used useful promoter, signal sequence for antibody secretion and the antibody gene to be expressed. Examples of promoters include lacz
15 promoter and araB promoter. Gene can be expressed according to the method of Ward, et al. (Nature (1989) 341, 544-546; FASEB J. (1992) 6, 2422-2427) in the case of using lacz promoter, or according to the method of Better, et al. (Science (1988) 240, 1041-1043) in the
20 case of using araB promoter.

 The pelB signal sequence (Lei, S.P. et al., J. Bacteriol. (1987) 169, 4379-4383) should be used as the signal sequence for antibody secretion in the case of producing in periplasm of E. coli. After isolating the
25 antibody produced in periplasm, the antibody is used after suitably refolding the antibody structure.

 Replication origins originating in SV40, poliovirus, adenovirus or bovine papilloma virus (BPV) and so forth can be used as replication origins. Moreover, in order
30 to amplify the number of gene copies in host cell systems, the expression vector can contain as selection marker aminoglycoside transferase (APH) gene, thymidine kinase (TK) gene, E. coli xanthine guanine phosphoribosyl transferase (Ecogpt) gene or dihydrofolate reductase
35 (dhfr) gene.

 An arbitrary expression system, such as a eucaryotic

required, and the details of the evaluation method are described in the specification of the patent application entitled "An animal model of a chronic hypercoagulable state and a method of generating the same" by the same
5 applicant as this invention. Specific examples of the evaluation method are described as Example 1 in this specification.

The result of the experiment that used the above humanized anti-human TF antibody version "i-b2" is shown
10 in Example 2 and Figures 11 to 13. According to this experiment, in the animal model system shown in Example 1, after the platelet count of the mice that were implanted with the tumor cell containing the human TF gene decreased to about half of that of the mice that
15 were not implanted with the same (5 to 6 weeks after implantation), 1 mg/kg of the humanized anti-human TF antibody version "i-b2" was repeatedly administered intravenously once a week, with a result that the platelet count was maintained at a level equal to that in
20 the mice that were not implanted with the tumor cell till the end of the experiment, i.e., three weeks after the start of the administration.

The administration of the humanized anti-human TF antibody of the present invention suppressed the increase
25 in the concentrations of soluble fibrin monomer complex (SFMC) and thrombin-antithrombin III complex (TAT). The result confirmed that the administration of anti-human TF antibody of the present invention prevents the persistence of a hypercoagulable state and maintains a
30 normal state.

8-2. Confirmation of the therapeutic effect on a hypercoagulable state resulting from infections

An elongation of prothrombin time, a decrease in the plasma concentration of fibrinogen, an increase in the
35 serum concentration of fibrin degradation products, and the like can be ascribed to the hypercoagulable state. The administration of anti-human TF antibody of the

Alternatively, the dosage of 0.01 to 100 mg/kg, preferably 0.1 to 10 mg/kg may be selected. However, the therapeutic agent containing anti-human TF antibody of the present invention is not limited to these dosages.

5 Preferably the method of administration is, but is not limited to, intravenous injection, intravenous drip, and the like.

 The therapeutic agent of the present invention comprising anti-human TF antibody as an active ingredient
10 may be formulated using a standard method (Remington's Pharmaceutical Science, the latest edition, Mark Publishing Company, Easton, USA), and may contain pharmaceutically acceptable carriers and/or additives.

 Examples of such carriers or additives include
15 water, a pharmaceutically acceptable organic solvent, collagen, polyvinyl alcohol, polyvinylpyrrolidone, a carboxyvinyl polymer, carboxymethylcellulose sodium, polyacrylic sodium, sodium alginate, water-soluble dextran, carboxymethyl starch sodium, pectin, methyl
20 cellulose, ethyl cellulose, xanthan gum, gum Arabic, casein, agar, polyethylene glycol, diglycerin, glycerin, propylene glycol, Vaseline, paraffin, stearyl alcohol, stearic acid, human serum albumin (HSA), mannitol, sorbitol, lactose, a pharmaceutically acceptable
25 surfactant and the like.

 Additives used are chosen from, but are not limited to, the above or combinations thereof, as appropriate, depending on the dosage form of the present invention. For example, when used as injections, purified anti-human
30 TF antibody may be dissolved in a solvent such as physiological saline, a buffer, and a glucose solution, to which an anti-adsorbent such as Tween 80, Tween 20, gelatin, and human serum albumin may be added. Alternatively, they may be lyophilized so as to be
35 dissolved and reconstituted into a dosage form before use. As the excipient for lyophilization, sugar alcohols and sugars such as mannitol and glucose may be used.

EXAMPLES

The present invention will now be explained more specifically with reference to the examples.

5 Example 1. Generation of experimental mice

A vector in which a gene encoding human tissue factor (SEQ ID NO: 103) had been inserted into an animal expression vector pCOS1 (hTF-pCOS1) was digested with a restriction enzyme PvuI and linearized, which was then
10 introduced into a human myeloma cell line KPMM2 (FERM P-14170) by electroporation.

pCOS1 was constructed by removing the antibody gene from HEF-PMh-gyl (WO 92/19759) by digesting with EcoRI and SmaI and then by ligating the EcoRI-NotI-BamHI
15 adaptor (Takara Shuzo). This was cultured in a RPMI1640 medium (containing 20% FCS hIL-6: 4 ng/ml) containing 1 mg/ml G418, and the cells that grew were confirmed to be the expression of human tissue factor using anti-human tissue factor antibody (American Diganostica) by flow
20 cytometry. This gave a cell line KPMM2/TF226 that has introduced a human tissue factor gene therein.

The parent strain (KPMM2/parent) before the introduction of the above human tissue factor gene and the gene-introduced strain KPMM2/TF226 were cultured in a
25 RPMI-1640 medium containing 4 ng/ml human IL-6 and 20% bovine fetal serum. The thus-grown KPMM2/TF226 cells and the parent KPMM2/parent cells, separately, were implanted subcutaneously at 1×10^7 cells into the flanks of SCID mice (available from CLEA Japan, male, 7-week old, mean
30 body weight: about 22 g), and changes with time in tumor volume, platelet counts in the blood, and plasma concentrations of human tissue factor, fibrinogen, soluble fibrin monomer complex (SFMC), and thrombin-antithrombin III complex (TAT) were investigated.

35 As a result, as shown in Figure 5, tumor volume increased with time in all mice. As shown in Figure 6, however, the plasma concentration of human tissue factor

confirming the persistence of a hypercoagulable state,
and thus from day 45 after the implanted 1 mg/kg of
humanized anti-human TF antibody version "i-b2" was
intravenously administered once a week. As a result, on
5 day 3 after the administration of humanized anti-human TF
antibody version "i-b2", platelet counts recovered to a
level higher than that in the non-tumor-implanted group,
and platelet counts were maintained at a level equal to
that in the non-tumor-implanted group during the period
10 from the start of the administration to week 3 when the
experiment ended.

On day 6 after the third administration of humanized
anti-human TF antibody version "i-b2", the plasma
concentrations of soluble fibrin monomer complex (sFMC)
15 and of thrombin-antithrombin III complex (TAT) were
determined, and it was found that the administration of
the antibody suppressed their increases (Figure 12 and
Figure 13). These results indicated that humanized anti-
human TF antibody version "i-b2" had the effect of stably
20 maintaining coagulation at a normal level, by a once-per-
week administration in the model in which a
hypercoagulable state persists for a long period.

Example 3.

LPS dissolved in physiological saline was
25 continuously injected into a vein at a dose of 1 mg/kg/hr
(2 ml/kg/hr) for 6 hours to cynomolgus monkeys (imported
from Chuang Primates Experimental Animal Research Center,
Nanning, the People's Republic of China, equal numbers of
males and females, estimated age: 5-7 years old, body
30 weight: 2.99-5.81 kg) under isoflurane anesthesia.
Intravenous administration of humanized anti-human TF
antibody "version i-b2" at 0.3 mg/kg (1 ml/kg) to monkeys
of the humanized anti-human TF antibody-administration
group, and that of a solvent (20 mM sodium acetate/150 mM
35 NaCl, pH 6.0) to monkeys of the control group were made
respectively 10 minutes before the start of continuous
injection of LPS.

At the end of continuous injection of LPS, citrated blood and normal blood were drawn via the catheter mounted to the femoral artery, and prothrombin time, plasma concentration of fibrinogen and serum concentration of fibrin degradation products were determined. As shown in Table 4, LPS injection resulted in the elongation of prothrombin time, decreases in plasma concentration of fibrinogen, and increases in serum concentration of fibrin degradation products, i.e. a hypercoagulable state, but in the monkeys that had received humanized anti-human TF antibody "version i-b2", these changes were strongly suppressed. These results reveal that humanized anti-human TF antibody "version i-b2" can suppress the hypercoagulable state resulting from infections.

Table 4
Effect of humanized anti-human TF antibody on hypercoagulability by LPS injection

	Control group (n=4)		Humanized anti-human TF antibody administration group (n=4)	
	Before LPS injection	After LPS injection	Before LPS injection	After LPS injection
Prothrombin time (seconds)	11.1 ± 0.3	15.8 ± 2.3	10.9 ± 0.3	11.7 ± 0.6
Plasma concentration of fibrinogen (mg/dl)	150 ± 20	90 ± 20	170 ± 10	160 ± 20
Serum concentration of fibrin degradation (μg/ml)	0 ± 0	43 ± 14	0 ± 0	8 ± 4

* Mean ± standard error

Example 4.

In a model of venous thrombosis induced by venostasis and a venous wall injury, the effect of humanized anti-human TF antibody on the venous thrombosis was evaluated. The venostasis was created by the

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TF antibody "version i-b2" was intravenously administered at a dose of 0.3 mg/kg and 1.5 mg/kg 2 hours before the start of the venous thrombus formation.

The results are shown in Table 5. The administration of humanized anti-human TF antibody led to the reduction in the weight of the clots formed. Therefore, these results indicate that humanized anti-human TF antibody has a prophylactic effect on the venous thrombus formation in this model.

10

Table 5

Effect of humanized anti-human TF antibody in the venous thrombosis cynomolgus monkey

	Test agent not administered (n=2)	Humanized anti-human TF antibody 0.3 mg/kg i.v. (n=2)	Humanized anti-human TF antibody 1.5 mg/kg i.v. (n=2)
Sum of the wet weight of venous clots in the left and right carotid veins (mg)	20.8 21.9	1.4 1.1	0.0 0.4
Mean	21.4	1.3	0.2

15

Example 5.

In a model of arterial thrombosis induced by angiostenosis and an arterial wall injury, the effect of humanized anti-human TF antibody on the arterial thrombosis was evaluated. The angiostenosis and the arterial wall injury were made by tightly ligating a blood vessel with a 20G needle pinched therein with its tip rounded and then removing the needle. This is a model that mimics angiostenosis due to arteriosclerosis and the arterial wall injury due to the plaque rupture.

25

Cynomolgus monkeys with an estimated age of 3-5 years old, weighing 3.55-3.99 kg (obtained from Chuang Primates Experimental Animal Research Center, the

People's Republic of China) were used in the arterial thrombosis model. The monkeys were anesthetized with ketamine hydrochloride (intramuscular administration) and butofanol (intramuscular administration) to expose the right common carotid artery. The probe of a doppler flowmeter was placed around the exposed blood vessel, and blood flow was monitored for about 5 minutes. After confirming the constant flow of the bloodstream, angiostenosis and the arterial wall injury were induced around the proximal site to the head side of the probe.

The blood flow was observed for the subsequent 15 minutes, and the time of vascular occlusion due to thrombus formation was determined. After loosening the ligation at the right common carotid artery, humanized anti-human TF antibody was administered to the monkeys of the antibody administration group. In the left common carotid artery as well, the time of vascular occlusion due to thrombus formation was determined. Humanized anti-human TF antibody "version i-b2" was intravenously administered at a dose of 0.3 mg/kg and 1.5 mg/kg one hour before the start of the thrombus formation at the left common carotid artery.

The results are shown in Table 6. The administration of humanized anti-human TF antibody led to the reduction in the time of vascular occlusion. Therefore, these results indicate that humanized anti-human TF antibody has a prophylactic effect on the arterial thrombus formation in this model.

minutes, 10 minutes before the vascular injury. The extracted artery was fixed in formalin, histological specimens were prepared, and stained with a HE stain and Elastica van Gieson stain, followed by image analysis to measure the area of the intima. As a result, as shown in Figure 7, humanized anti-human TF antibody "version i-b2" strongly suppressed the hypertrophy of the intima. This indicated that humanized anti-human TF antibody "version i-b2" prevents the narrowing of the area of the lumen during the remote period by suppressing the growth of the blood vessel tissue itself, suggesting that it can effectively prevent restenosis.

Table 7

	Animal No.	Non-injured blood vessel	Injured blood vessel
		Area of media (mm ²)	Area of media (mm ²)
Control group	1	1.06	2.15 (203%)
	2	0.74	1.45 (196%)
	3	0.82	1.78 (217%)
Anti-human TF antibody	4	0.75	1.15 (153%)
	5	0.78	0.96 (123%)
	6	0.86	0.98 (114%)

(Percentage relative to the non-injured side)

Example 7.

The effect of humanized anti-human TF antibody "version i-b2" and low molecular weight heparin was investigated in the model described in Example 1. Six to seven weeks after the grafting of KPMM2/TF226 to SCID mice (CLEA Japan, male, 7 weeks old, mean body weight: about 24 g), platelet counts reduced to about half that of the non-tumor-grafted group, confirming the persistence of the hypercoagulable state. Hence, from day 49 after the grafting 1 mg/kg of humanized anti-human TF antibody version "i-b2" was intravenously administered or low molecular weight heparin at 601.5 IU/kg, 1900.3 IU/kg, and 6487.3 IU/kg was continuously administered by subcutaneously embedding an osmotic pump that permits

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changed to 0.3 M, and shTF was eluted from the column.
To the shTF fraction obtained, ammonium sulfate was added
to a final concentration of 2.5 M, and was centrifuged
(10,000 rpm, 20 minutes) to precipitate the contaminating
5 proteins. The supernatant was added to Butyl TOYOPEARL
(30 ml, TOSOH), and then was washed with a 50 mM Tris-HCl
buffer (pH 6.8) containing 2.5 M ammonium sulfate. In
the 50 mM Tris-HCl buffer (pH 6.8), the concentration of
ammonium sulfate was linearly reduced from 2.5 M to 0 M
10 to permit the elution of shTF. The peak fractions
containing shTF were concentrated by the Centri-Prep 10
(Amicon). The concentrate was added to the TSKgel
G3000SWG column (21.5 x 600 mm, TOSOH) equilibrated with
a 20 mM Tris-HCl buffer (pH 7.0) containing 150 mM NaCl,
15 and the peak fraction of shTF was collected. It was
filter sterilized with a 0.22 µm membrane filter and the
product was set as the soluble human TF (shTF). The
concentration of the sample was calculated assuming that
the molar extinction coefficient of the sample $\epsilon = 40,130$
20 and molecular weight = 43,210.

Reference Example 2. Preparation of anti-TF
monoclonal antibody

1. Purification of human TF

The purification of TF from human placenta was
25 carried out according to the method of Ito (Ito, T. et
al., J. Biol. Chem., 114: 691-696, 1993). Thus, human
placenta was homogenized in Tris buffered saline (TBS,
pH 7.5) containing 1.0 mM benzamidine hydrochloride, 1 mM
phenylmethylsulfonyl fluoride, 1 mM diisopropylfluoro
30 phosphate, and 0.02% sodium azide, and then the
precipitate was defatted with cold acetone. The defatted
powder obtained was suspended in the above buffer
containing 2% Triton X-100 to solubilize TF.

The supernatant was subjected to affinity
35 chromatography using Concanavalin A-Sepharose 4B column
(Pharmacia) and anti-TF antibody-bound Sepharose 4B

column (Pharmacia), and purified TF was obtained. This was concentrated with an ultrafiltration membrane (PM-10, Amicon) and was stored as the purified sample at 4°C.

5 TF content in the purified sample was quantitated by Sandwich ELISA that combined a commercially available anti-TF monoclonal antibody (American Diagnostica) and polyclonal antibody (American Diagnostica) with recombinant TF as a standard.

10 The purity in the purified sample was confirmed by subjecting the sample to SDS-PAGE using a 4-20% density gradient polyacrylamide gel, and silver-staining the product.

2. Immunization and the preparation of hybridoma

After mixing the purified human TF (about 70 µg/ml) with an equal volume of Freund's complete adjuvant (Difco), it was immunized subcutaneously into the abdomen of 5-week old Balb/c male mice (Nippon Charles River) at 10 µg TF/mouse. On day 12, 18, and 25, TF mixed with Freund's incomplete adjuvant was subcutaneously boosted at 5 µg/mouse TF, and as a final immunization the TF solution diluted with PBS was intraperitoneally given at 5 µg/mouse on day 32.

25 Three days after the final immunization, the spleen cells were prepared from four mice, and were fused to the mouse myeloma cell line P3U1 at 1/5 cell count thereof by the polyethylene glycol method. The fused cells were suspended into the RPMI-1640 medium (hereinafter referred to as RPMI-medium) (Lifetech Oriental) containing 10% fetal bovine serum, which was inoculated in 400 wells per mouse (about 400 cells/well) of a 96-well plate. On day 1, 2, 3, and 5 after the fusion, half the volume of the medium was exchanged with the RPMI-medium (hereinafter referred to as HAT-medium) containing HAT (Dainippon Seiyaku) and condimed H1 (Boehringer Mannheim GmbH) to perform HAT selection of the hybridoma.

35 The hybridomas selected by the screening method

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described below were cloned by conducting limiting dilution twice.

For the limiting dilution, 0.8 cells was inoculated per well in two 96-well plates. For the wells in which single colony was confirmed by microscopic examination, clones were selected by the following measurement of the binding activity to TF and neutralizing activity against TF. The clones obtained were acclimated from the HAT-medium to the RPMI-medium. After the absence of reduction in antibody production ability due to acclimation was confirmed, limiting dilution was performed again for complete cloning. By the foregoing procedure, hybridomas that produce six antibodies (ATR-2, 3, 4, 5, 7, and 8) that strongly inhibit the binding of TF/Factor VIIa complex and Factor X were established.

3. Ascites formation and antibody purification

The ascites formation of the established hybridomas were carried out according to the standard method. Thus, 10^6 hybridomas that were subcultured in vitro were intraperitoneally grafted into BALB/c male mice that had previously received twice intravenous administration of mineral oil. Ascites was collected from the mice that showed a bloated abdomen 1-2 weeks after the grafting.

The purification of antibody from ascites was carried out using the ConSepLC100 system (Millipore) equipped with the Protein A column (Nippon Gaishi).

4. Cell-ELISA

Human bladder carcinoma cells J82 (Fair D. S. et al., J. Biol. Chem., 262: 11692-11698, 1987) that are known to express TF at a high level were obtained from ATCC, and subcultured and maintained in the RPMI-medium under the condition of 37°C, 5% CO₂, and 100% humidity.

Cell-ELISA plates were prepared by inoculating J82 cells to a 96-well plate at 10^5 cells/well, culturing for one day under the above condition, removing the medium and then washing twice with phosphate buffered saline (PBS), adding a 4% paraformaldehyde solution (PFA), and

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allowing to stand on ice for 10 minutes for immobilization. After PFA was removed, the plate was washed with PBS, the Tris buffer (Blocking buffer) containing 1% BSA and 0.02% sodium azide was added thereto, and the plate was stored at 4°C until use.

Cell-ELISA was carried out in the following manner. Thus, the Blocking buffer was removed from the plate prepared as above, to which an anti-TF antibody solution or a hybridoma culture supernatant was added and was reacted at room temperature for 1.5 hours. After washing with PBS containing 0.05% Tween 20, alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) (Zymed) was reacted for 1 hour. After washing, 1 mg/ml p-nitrophenyl phosphate disodium (Sigma) was added, and one hour later absorbance at 405/655 nm was measured to determine the amount of anti-TF antibody that bound to the J82 cells.

5. Assay system of neutralizing activity against TF with Factor Xa activity as an index

To 50 µl of Tris buffered saline (TBS: pH 7.6) containing 5 mM CaCl₂ and 0.1% bovine serum albumin, 10 µl of a human placenta-derived thromboplastin solution (5 mg/ml) (Thromborel S) (Boehring) and 10 µl of a Factor VIIa solution (82.5 ng/ml) (American Diagnostics) were added, and reacted at room temperature for 1 hour to permit the formation of the TF/Factor VIIa complex. After 10 µl of a predetermined concentration of a diluted anti-TF antibody solution or the hybridoma culture supernatant and 10 µl of a Factor X solution (3.245 µg/ml) (Celsus Laboratories) were added and reacted for 45 minutes, 10 µl of 0.5 M EDTA was added to stop the reaction. Fifty µl of 2 mM S-2222 solution (Daiichi Kagaku Yakuhin) was added thereto, and changes in absorbance at 405/655 nm over 30 minutes were measured and was set as the Factor X-producing activity of TF. In this method, the activity of antibody that inhibits the

mRNA was purified by the oligo (dT)-cellulose spun column, followed by ethanol precipitation. The mRNA precipitate was dissolved in the elution buffer.

5 (2) Preparation and amplification of cDNA of the gene encoding a mouse antibody V region

(i) Cloning of H chain V region cDNA

The cloning of the gene encoding the H chain V region of a mouse monoclonal antibody against human TF was carried out using the 5'-RACE method (Frohman, M.A. 10 et al., Proc. Natl. Acad. Sci. USA 85: 8998-9002, 1988; Belyavsky, A. et al., Nucleic Acids Res. 17: 2919-2932, 1989). For the 5'-RACE method, the Marathon cDNA Amplification Kit (CLONTECH) was used and the procedure carried out according to the instructions attached to the 15 kit.

Using about 1 µg of mRNA prepared as above as a template, the cDNA synthesis primer attached to the kit was added, which was reacted with a reverse transcriptase at 42°C for 60 minutes to effect reverse transcription to 20 cDNA. This was reacted with DNA polymerase I, DNA ligase, and RNaseH at 16°C for 1.5 hour, and with T4 DNA polymerase at 16°C for 45 minutes thereby to synthesize a double stranded cDNA. The double stranded cDNA was extracted with phenol and chloroform, and recovered by 25 ethanol precipitation.

By overnight reaction with T4 DNA ligase at 16°C, a cDNA adapter was ligated to both ends of the double stranded cDNA. The reaction mixture was diluted 50-fold with a 10 mM Tricine-KOH (pH 8.5) containing 0.1 mM EDTA. 30 Using this as a template, the gene encoding the H chain V region was amplified by PCR. The adapter primer 1 attached to the kit was used for the 5'-end primer, and for the 3'-end primer the MHC-G1 primer (SEQ ID NO: 1) (S. T. Jones, et al., Biotechnology, 9: 88-89, 1991) were 35 used.

PCR solutions for the ATR-5 antibody H chain V

region contained, in 100 μ l, 120 mM Tris-HCl (pH 8.0), 10 mM KCl, 6 mM $(\text{NH}_4)_2\text{SO}_4$, 0.1% Triton X-100, 0.001% BSA, 0.2 mM dNTPs (dATP, dGTP, dCTP, dTTP), 1 mM MgCl_2 , 2.5 units of KOD DNA polymerase (Toyo Boseki), 30-50 pmole of
5 adapter primer 1, as well as MHC-G1 primer, and 1-5 μ l of a reaction mixture of cDNA to which the cDNA adapter was ligated.

All PCRs were carried out using the DNA Thermal
Cycler 480 (Perkin-Elmer), and the PCR was performed for
10 thirty cycles at a temperature cycle of 94°C for 30 seconds, 55°C for 30 seconds, and 74°C for 1 minute.

(ii) Cloning of L chain V region cDNA

The cloning of the gene encoding the L chain V region of a mouse monoclonal antibody against human TF
15 was carried out using the 5'-RACE method (Frohman, M.A. et al., Proc. Natl. Acad. Sci. USA 85: 8998-9002, 1988; Belyavsky, A. et al., Nucleic Acids Res. 17: 2919-2932, 1989). For the 5'-RACE method, the Marathon cDNA Amplification Kit (CLONTECH) was used and carried out
20 according to the instructions attached to the kit. Using about 1 μ g of mRNA prepared as above as a template, the cDNA synthesis primer was added, which was reacted with a reverse transcriptase at 42°C for 60 minutes to effect reverse transcription to cDNA.

25 This was reacted with DNA polymerase I, DNA ligase, and RNaseH at 16°C for 1.5 hour, and with T4 DNA polymerase at 16°C for 45 minutes thereby to synthesize a double stranded cDNA. The double stranded cDNA was extracted with phenol and chloroform, and recovered by
30 ethanol precipitation. By overnight reaction with T4 DNA ligase at 16°C, a cDNA adapter was ligated to both ends of the double stranded cDNA. The reaction mixture was diluted 50-fold with a 10 mM Tricine-KOH (pH 8.5) containing 0.1 mM EDTA. Using this as a template, the
35 gene encoding the L chain V region was amplified by PCR. The adapter primer 1 was used for the 5'-end primer, and

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JM109 competent cells (Nippongene) and was incubated for 30 minutes on ice and for 1 minute at 42°C.

Then, 300 µl of the Hi-Competence Broth (Nippongene) was added thereto, incubated at 37°C for 1 hour. Then,
5 Escherichia coli was plated on a LB agar medium
(Molecular Cloning: A Laboratory Manual, Sambrook, et al., Cold Spring Harbor Laboratory Press, 1989)
containing 100 µg/ml ampicillin (hereinafter referred to as LBA agar medium), and incubated overnight at 37°C to
10 obtain an E. coli transformant.

The transformant was cultured overnight in 3 ml or 4 ml of a LB medium containing 50 µg/ml ampicillin
(hereinafter referred to as LBA medium) at 37°C, and from the cell fractions, plasmid DNA was prepared using the
15 QIAprep Spin Plasmid Kit (QIAGEN), and then the nucleotide sequence was determined.

(4) Determination of the nucleotide sequence of the gene encoding a mouse antibody V region

The nucleotide sequence of the cDNA coding region in
20 the above plasmid was determined using the Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer) by the DNA Sequencer 373A (Perkin-Elmer). As the sequencing primer, M13 Primer M4 (Takara Shuzo) (SEQ ID NO: 3) and M13 Primer RV (Takara Shuzo) (SEQ ID NO: 4) were used,
25 and the sequence was determined by confirming the nucleotide sequence in both directions.

Thus obtained plasmids containing the gene encoding the mouse H chain V region derived from the hybridoma ATR-5 was designated as ATR-5Hv/pUC19, and the thus
30 obtained plasmids containing the gene encoding a mouse L chain V region derived from the hybridoma ATR-5 was designated as ATR-5Lv/pUC19. The nucleotide sequences of the genes encoding the H chain V region of each mouse antibody contained in the plasmid ATR-5Hv/pUC19
35 (including the corresponding amino acid sequences) is shown in SEQ ID NO: 5 and 99, respectively, and the

nucleotide sequences of the genes encoding the L chain V region of each mouse antibody contained in the plasmid ATR-5Lv/pUC19 (including the corresponding amino acid sequences) is shown in SEQ ID NO: 6 and 100, respectively.

Reference Example 4. Construction of chimeric antibody

A chimeric ATR-5 antibody was generated in which the mouse ATR-5 antibody V region was ligated to the human antibody C region. A chimeric antibody expression vector was constructed by ligating the gene encoding the ATR-5 antibody V region to an expression vector encoding the human antibody C region.

(1) Construction of a chimeric antibody H chain V region

The ATR-5 antibody H chain V region was modified by the PCR method in order to ligate it to an expression vector encoding the human antibody H chain C region. The 5'-end primer ch5HS (SEQ ID NO: 7) was designed so as to hybridize the 5'-end of DNA encoding the V region and to have the Kozak consensus sequence (Kozak, M. et al., J. Mol. Biol. 196: 947-950, 1987) and a recognition sequence of the restriction enzyme SalI. The 3'-end primer ch5HA (SEQ ID NO: 8) was designed so as to hybridize 3'-end of DNA encoding the J region and to have a recognition sequence of the restriction enzyme NheI.

The PCR solutions contained, in 100 μ l, 120 mM Tris-HCl (pH 8.0), 10 mM KCl, 6 mM $(\text{NH}_4)_2\text{SO}_4$, 0.1% Triton X-100, 0.001% BSA, 0.2 mM dNTPs (dATP, dGTP, dCTP, dTTP), 1 mM MgCl_2 , 2.5 units of KOD DNA polymerase (Toyo Boseki), 50 pmole of the ch5HS primer and the ch5HA primer, as well as 1 μ l of the plasmid ATR5Hv/pUC19 as a template DNA. For PCR, the DNA Thermal Cycler 480 (Perkin-Elmer) was used, and the PCR was performed for thirty cycles at a temperature cycle of 94°C for 30 seconds, 55°C for 30 seconds, and 74°C for 1 minute.

primer, M13 Primer M4 (Takara Shuzo) (SEQ ID NO: 3) and
M13 Primer RV (Takara Shuzo) (SEQ ID NO: 4) were used,
and the sequence was determined by confirming the
nucleotide sequence in both directions. The plasmid that
5 contains the gene encoding the ATR-5 antibody H chain V
region, a SalI recognition sequence and the Kozak
consensus sequence at the 5'-end, and a NheI recognition
sequence at the 3'-end was designated as chATR5Hv/CVIDEC.

(2) Construction of a chimeric antibody L chain V
10 region

The ATR-5 antibody L chain V region was modified by
the PCR method in order to ligate it to an expression
vector encoding the human antibody L chain C region. The
5'-end primer ch5LS (SEQ ID NO: 9) was designed so as to
15 hybridize to the 5'-end of the DNA encoding the V region
and to have the Kozak consensus sequence (Kozak, M. et
al., J. Mol. Biol. 196: 947-950, 1987) and a recognition
sequence of the restriction enzyme BglII. The 3'-end
primer ch5LA (SEQ ID NO: 10) was designed so as to
20 hybridize to the 3'-end of the DNA encoding the J region
and to have a recognition sequence of the restriction
enzyme SphI.

The PCR solutions contained, in 100 μ l, 120 mM Tris-
HCl (pH 8.0), 10 mM KCl, 6 mM $(\text{NH}_4)_2\text{SO}_4$, 0.1% Triton X-
25 100, 0.001% BSA, 0.2 mM dNTPs (dATP, dGTP, dCTP, dTTP), 1
mM MgCl_2 , 2.5 units of KOD DNA polymerase (Toyo Boseki),
50 pmole of the ch5LS primer and the ch5LA primer, as
well as 1 μ l of the plasmid ATR5Lv/pUC19 as a template
DNA. For PCR the DNA Thermal Cycler 480 (Perkin-Elmer)
30 was used, and the PCR was performed for thirty cycles at
a temperature cycle of 94°C for 30 seconds, 55°C for 30
seconds, and 74°C for 1 minute.

The PCR reaction mixture was extracted with phenol
and chloroform, and the amplified DNA fragments were
35 recovered by ethanol precipitation. The DNA fragments
were digested with the restriction enzyme SphI (Takara

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Shuzo) at 37°C for 1 hour, and then with the restriction enzyme BglII (Takara Shuzo) at 37°C for 1 hour. The digestion mixture was separated by agarose gel electrophoresis using a 3% NuSieve GTG agarose (FMC BioProducts), and the agarose strips containing about 400 bp long DNA fragments were excised. The agarose strips were extracted with phenol and chloroform, the DNA fragments were precipitated with ethanol, which were then dissolved in 20 µl of TE.

The gene fragment prepared as above encoding the mouse L chain V region and the CVIDEC vector prepared by digesting with SplI and BglII were ligated using the DNA ligation kit ver.2 (Takara Shuzo) by reacting at 16°C for 1 hour according to the instructions attached to the kit.

The ligation mixture was added to 100 µl of E. coli JM109 competent cells (Nippongene) and was incubated for 30 minutes on ice and for 1 minute at 42°C. Then, 300 µl of the Hi-Competence Broth (Nippongene) was added thereto, incubated at 37°C for 1 hour, and then the E. coli was plated on a 100 µg/ml LBA agar medium and incubated overnight at 37°C to obtain an E. coli transformant. The transformant was cultured overnight at 37°C in 3 ml of the LBA medium, and from the cell fractions, plasmid DNA was prepared using the QIAprep Spin Plasmid Kit (QIAGEN).

The nucleotide sequence of the cDNA coding region in the plasmid was determined using the Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer) by the DNA Sequencer 373A (Perkin-Elmer). As the sequencing primer, M13 Primer M4 (Takara Shuzo) and M13 Primer RV (Takara Shuzo) were used, and the sequence was determined by confirming the nucleotide sequence in both directions. The plasmid that contains the gene encoding the ATR-5 antibody L chain V region and that has a BglII recognition sequence and the Kozak consensus sequence at the 5'-end and a SplI recognition sequence at the 3'-end

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fragments were precipitated with ethanol, which were then dissolved in 20 μ l of TE.

5 The SalI-NheI DNA fragment prepared as above containing the gene encoding the H chain V region and N5KG1(V) or N5KG4P digested with SalI and NheI were ligated using the DNA ligation kit ver.2 (Takara Shuzo) by reacting at 16°C for 1 hour according to the attached instructions.

10 The ligation mixture was added to 100 μ l of E. coli JM109 competent cells (Nippongene) and was incubated for 30 minutes on ice and for 1 minute at 42°C. Then, 300 μ l of the Hi-Competence Broth (Nippongene) was added thereto, incubated at 37°C for 1 hour, and then the E. coli was plated on a 100 μ g/ml LBA agar medium and
15 incubated overnight at 37°C to obtain an E. coli transformant. The transformant was cultured overnight at 37°C in 3 ml of the LBA medium, and from the cell fractions, plasmid DNA was prepared using the QIAprep Spin Plasmid Kit (QIAGEN). These plasmids containing the
20 genes encoding the chimeric ATR-5 antibody H chain were designated as chATR5Hv/N5KG1(V) and chATR5Hv/N5KG4P, respectively.

(ii) Introduction of the L chain V region

25 The plasmid chATR5Lv/CVIDEC was digested with the restriction enzymes BglII (Takara Shuzo) and SphI (Takara Shuzo) at 37°C for 1.5 hour. The digestion mixture was separated by agarose gel electrophoresis using 1.5% NuSieve GTG agarose (FMC BioProducts), and the agarose strips containing about 400 bp long DNA fragments were
30 excised. The agarose strips were extracted with phenol and chloroform, and the DNA fragments were precipitated with ethanol, which were then dissolved in 20 μ l of TE.

35 The plasmids chATR5Hv/N5KG1(V) and chATR5Hv/N5KG4P were digested with the restriction enzymes BglII (Takara Shuzo) and SphI (Takara Shuzo) at 37°C for 1.5 hour. The

digestion mixture was separated by agarose gel electrophoresis using 1.5% NuSieve GTG agarose (FMC BioProducts), and the agarose strips containing about 9400 bp long DNA fragments were excised. The agarose strips were extracted with phenol and chloroform, DNA fragments were precipitated with ethanol, which were then dissolved in 20 µl of TE.

The SphI-BglII DNA fragment prepared as above containing the gene encoding the L chain V region and chATR5Hv/N5KG1(V) or chATR5Hv/N5KG4P digested with SphI and BglII were ligated using the DNA ligation kit ver.2 (Takara Shuzo) by reacting at 16°C for 1 hour according to the attached instructions.

The ligation mixture was added to 100 µl of E. coli JM109 competent cells (Nippongene) and was incubated for 30 minutes on ice and for 1 minute at 42°C. Then, 300 µl of the Hi-Competence Broth (Nippongene) was added thereto, incubated at 37°C for 1 hour, and then the E. coli was plated on a 100 µg/ml LBA agar medium and incubated overnight at 37°C to obtain an E. coli transformant. The transformant was cultured overnight at 37°C in 1 l of the 2xYT medium containing 50 µg/ml ampicillin, and from the cell fractions, plasmid DNA was prepared using the Plasmid Maxi Kit (QIAGEN). These plasmids containing the gene encoding the chimeric ATR-5 antibody were designated as chATR5/N5KG1(V) and chATR5/N5KG4P, respectively.

(4) Transfection into COS-7 cells

In order to evaluate the activity of binding to the antigen and the neutralizing activity of chimeric antibody, the above expression plasmid was transfected to COS-7 cells and the antibody was transiently expressed.

The plasmid chATR5/N5KG1(V) or chATR5/N5KG4P was transduced into COS-7 cells by electroporation using the Gene Pulser instrument (Bio Rad). Fifty µg of the

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plasmid was added to 0.78 ml of the COS-7 cells suspended in the Dulbecco PBS (-) (hereinafter referred to as PBS) at a cell concentration of 1×10^7 cells/ml, which was subjected to pulses of 1,500 V and 25 μ F capacity.

5 After 10 minutes of the recovery period at room temperature, the electroporated cells were suspended in a DMEM medium containing 5% Ultra low IgG fetal bovine serum (GIBCO), and cultured using a 10 cm culture dish in a 5% CO₂ incubator. After culturing for 24 hours, the
10 culture supernatant was aspirated off, and then a serum-free medium HBCHO (Irvine Scientific) was added. After further culturing for 72 hours, the culture supernatant was collected and centrifuged to remove cell debris.

(5) Purification of antibody

15 From the culture supernatant of the COS-7 cells, chimeric antibody was purified using the rProtein A Sepharose Fast Flow (Pharmacia Biotech) as follows.

One ml of rProtein A Sepharose Fast Flow was filled into a column and the column was equilibrated by 10
20 volumes of TBS. The culture supernatant of COS-7 cells was applied to the equilibrated column, which was then washed with 10 volumes of TBS.

The adsorbed antibody fraction was then eluted by 13.5 ml of 2.5 mM HCl (pH 3.0), and the eluate was
25 immediately neutralized by adding 1.5 ml of 1 M Tris-HCl (pH 8.0).

By performing ultrafiltration twice for the purified antibody fraction using the Centriprep 100 (Amicon), the solvent was replaced to 50 mM Tris-HCl (pH 7.6)
30 containing 150 mM NaCl (hereinafter referred to as TBS), and was finally concentrated to about 1.5 ml.

(6) Establishment of a stably-producing CHO cell line

In order to establish a cell line that stably
35 produces chimeric antibody, the above expression plasmid was introduced into CHO cells (DG44) acclimated to the

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CHO-S-SFMII serum-free medium (GIBCO).

The plasmid chATR5/N5KG1(V) or chATR5/N5KG4P was
cleaved with the restriction enzyme SspI (Takara Shuzo)
to linearize DNA, and after extraction with phenol and
5 chloroform, DNA was recovered by ethanol precipitation.
The linearized plasmid was transduced into the DG44 cells
by electroporation using the Gene Pulser instrument (Bio
Rad). Ten µg of the plasmid was added to 0.78 ml of DG44
cells suspended in PBS at a cell concentration of 1×10^7
10 cells/ml, which was subjected to pulses of 1,500 V and 25
µF capacity.

After 10 minutes of the recovery period at room
temperature, the electroporated cells were suspended in a
CHO-S-SFMII medium (GIBCO) containing
15 hypoxanthine/thymidine (GIBCO), and cultured using two
96-well plates (Falcon) in a 5% CO₂ incubator. On the
day after the start of culturing, the medium was changed
to a selection medium containing the CHO-S-SFMII medium
(GIBCO) containing hypoxanthine/thymidine (GIBCO) and 500
20 µg/ml GENETICIN (G418Sulfate, GIBCO) to select cells into
which the antibody gene had been introduced. After
changing the selection medium, the cells were examined
under a microscope about two weeks later. After a
favorable cell growth was observed, the amount of
25 antibody produced was measured by the ELISA described
below for determining antibody concentration, and cells
having a high production yield of antibody were selected.

Reference Example 5. Construction of humanized
antibody

- 30 (1) Construction of humanized antibody H chain
(i) Construction of the humanized H chain version
"a"

Humanized ATR-5 antibody H chain was generated using
CDR-grafting by the PCR method. In order to generate the
35 humanized antibody H chain version "a" having the FRs
derived from human antibody L39130 (DDBJ, Gao L. et al.,

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acid sequence of version "b" is also shown in SEQ ID NO: 29.

F3NMFS and F3NMFA, and F3NMBS and F3NMBA were annealed, and were digested with BalI and XhoI, and NcoI and XhoI, respectively. They were introduced to the plasmid hATR5Hva/CVIDEC (BalI/NcoI) prepared by digesting with BalI and NcoI, and the nucleotide sequence was determined. The plasmid having the correct sequence was designated as hATR5Hvc/CVIDEC. The nucleotide sequence and the corresponding amino acid sequence of the humanized H chain version "c" contained in the plasmid hATR5Hvc/CVIDEC are shown in SEQ ID NO: 30. The amino acid sequence of version "c" is also shown in SEQ ID NO: 31.

(iii) Construction of humanized H chain versions "d" and "e"

Versions "d" and "e" were generated by replacing the FR3 of version "a" with the FR3 derived from another human antibody using the FR-shuffling method. In order to replace the FR3 in version "d" with one derived from human antibody M62723 (DDBJ, Pascual V. et al., J. Clin. Invest., 86: 1320-1328, 1990), four DNA primers encoding the FR3 were generated. The FR-shuffling primer F3EPS (SEQ ID NO: 32) has a sense DNA sequence and F3EPA (SEQ ID NO: 33) has an antisense DNA sequence, and the 3'-end of the primers has a complementary sequence of 18 bp.

Exogenous primers F3PrS (SEQ ID NO: 34) and F3PrA (SEQ ID NO: 35) have a homology with the FR-shuffling primers F3EPS and F3EPA, and can also be used for other FR3's FR-shuffling. In order to replace the FR3 in version "e" with one derived from the human antibody Z80844 (DDBJ, Thomsett AR. et al., unpublished), two DNA primers encoding the FR3 were generated. The FR-shuffling primers F3VHS (SEQ ID NO: 36) has a sense DNA sequence and F3VHA (SEQ ID NO: 37) has an antisense DNA sequence, and the 3'-end of the primers has a complementary sequence of 18 bp. F3EPS, F3EPA, F3PrS,

F3PrA, F3VHS and F3VHA were synthesized by Pharmacia Biotech.

PCR was performed using the KOD DNA polymerase (Toyo Boseki) using the attached buffer under the condition of
5 containing 5 μ l each of 1 μ M FR-shuffling primers F3EPS and F3EPA, or F3VHS and F3VHA, 0.2 mM dNTPs, 1.0 mM $MgCl_2$, and 2.5 units of KOD DNA polymerase in 100 μ l of the reaction mixture, for 5 cycles at a temperature cycle of 94°C for 30 seconds, 50°C for 1 minute, and 74°C for 1
10 minute. After further addition of 100 pmole of exogenous primers F3PrS and F3PrA, PCR was performed for 25 cycles with the same temperature cycle.

DNA fragments amplified by the PCR method were separated by agarose gel electrophoresis using a 2% Nu
15 Sieve GTG agarose (FMC BioProducts). The agarose strips containing about 424 bp long DNA fragments were excised, to which 3 volumes (ml/g) of TE was added, and then were extracted with phenol, phenol/chloroform, and chloroform to purify the DNA fragments. After precipitating the
20 purified DNA with ethanol, one third the volume thereof was dissolved in 14 μ l of water. The PCR reaction mixture obtained was digested with *Bal*I and *Nco*I, and was introduced to the plasmid hATR5Hva/CVIDEC (*Bal*I/*Nco*I) prepared by digesting with *Bal*I and *Nco*I, and the
25 nucleotide sequence was determined.

The plasmids having the correct sequence were designated as hATR5Hvd/CVIDEC and hATR5Hve/CVIDEC. The nucleotide sequence and the corresponding amino acid sequence of the humanized H chain version "d" contained
30 in the plasmid hATR5Hvd/CVIDEC are shown in SEQ ID NO: 38, and the amino acid sequence of version "d" is also shown in SEQ ID NO: 39. The nucleotide sequence and the corresponding amino acid sequence of the humanized H chain version "e" contained in the plasmid
35 hATR5Hve/CVIDEC are shown in SEQ ID NO: 40, and the amino acid sequence of version "e" is also shown in SEQ ID NO:

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The plasmids having the correct sequence were designated as hATR5Hvi/CVIDEC and hATR5Hvj/CVIDEC. The nucleotide sequence and the corresponding amino acid sequence of the humanized H chain version "i" contained in the plasmid hATR5Hvi/CVIDEC, and the amino acid sequence of version "i" are shown in SEQ ID NO: 58 and 59. The nucleotide sequence and the corresponding amino acid sequence of the humanized H chain version "j" contained in the plasmid hATR5Hvj/CVIDEC, and the amino acid sequence of version "j" are shown in SEQ ID NO: 60 and 61.

(vii) Construction of humanized H chain versions "b1" and "d1"

Versions "b1" and "d1" were generated by replacing the FR2 of versions "b" and "d" with the FR2 derived from another human antibody using the FR-shuffling method. In order to replace the FR2 with one derived from the human antibody P01742 (SWISS-PROT, Cunningham BA. et al., Biochemistry, 9: 3161-3170, 1970), two DNA primers encoding the FR2 were synthesized. The FR-shuffling vector F2MPS (SEQ ID NO: 62) has a sense DNA sequence and F2MPA (SEQ ID NO: 63) has an antisense DNA sequence. They also have a sequence complementary to each other, and have recognition sequences of EcoT221 and BalI on both ends thereof.

F2MPS and F2MPA were synthesized and purified by Pharmacia Biotech. F2MPS and F2MPA were annealed and were digested with EcoT221 and BalI. They were introduced to plasmids hATR5Hvb/CVIDEC (EcoT221/BalI) and hATR5Hvd/CVIDEC (EcoT221/BalI) prepared by digesting with EcoT221 and BalI, and the nucleotide sequence was determined. The plasmids having the correct sequence were designated as hATR5Hvb1/CVIDEC and hATR5Hvd1/CVIDEC. The nucleotide sequence and the corresponding amino acid sequence of the humanized H chain version "b1" contained in the plasmid hATR5Hvb1/CVIDEC, and the amino acid sequence of version "b1" are shown in SEQ ID NO: 64 and

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65. The nucleotide sequence and the corresponding amino acid sequence of the humanized H chain version "d1" contained in the plasmid hATR5Hvd1/CVIDEC, and the amino acid sequence of version "d1" are shown in SEQ ID NO: 66 and 67.

(viii) Construction of humanized H chain versions "b3" and "d3"

Versions "b3" and "d3" were generated by replacing the FR2 of versions "b" and "d" with the FR2 derived from another human antibody using the FR-shuffling method. In order to replace the FR2 with one derived from the human antibody Z80844 (DDDJ, Thomsett AR. et al., unpublished), two DNA primers encoding the FR2 were synthesized. The FR-shuffling vector F2VHS (SEQ ID NO: 68) has a sense DNA sequence and F2VHA (SEQ ID NO: 69) has an antisense DNA sequence. They also have a sequence complementary to each other, and have recognition sequences of EcoT221 and BalI on both ends thereof. The synthesis and purification of F2VHS and F2VHA was referred to Pharmacia Biotech.

F2VHS and F2VHA were annealed and were digested with EcoT221 and BalI. They were introduced to plasmids hATR5Hvb/CVIDEC (EcoT221/BalI) and hATR5Hvd/CVIDEC (EcoT221/BalI) prepared by digesting with EcoT221 and BalI, and the nucleotide sequence was determined. The plasmids having the correct sequence were designated as hATR5Hvb3/CVIDEC and hATR5Hvd3/CVIDEC. The nucleotide sequence and the corresponding amino acid sequence of the humanized H chain version "b3" contained in the plasmid hATR5Hvb3/CVIDEC, and the amino acid sequence of version "b3" are shown in SEQ ID NO: 70 and 71. The nucleotide sequence and the corresponding amino acid sequence of the humanized H chain version "d3" contained in the plasmid hATR5Hvd3/CVIDEC, and the amino acid sequence of version "d3" are shown in SEQ ID NO: 72 and 73.

(2) Construction of a humanized antibody L chain V region

were extracted with phenol and chloroform, DNA fragments were recovered by ethanol precipitation. The recovered DNA fragments were digested with the restriction enzymes SphI (Takara Shuzo) and BglII (Takara Shuzo) at 37°C for 4 hours. The digestion mixture was extracted with phenol and chloroform, and after the ethanol precipitation of the DNA fragments, they were dissolved in 10 µl of TE. The SphI-BglII DNA fragment prepared as above encoding the humanized L chain V region and the CVIDEC vector prepared by digesting with SphI and BglII were ligated using the DNA ligation kit ver.2 (Takara Shuzo) by reacting at 16°C for 1 hour according to the instructions attached to the kit.

The ligation mixture was added to 100 µl of E. coli JM109 competent cells (Nippongene) and was incubated for 30 minutes on ice and for 1 minute at 42°C. Then, 300 µl of the Hi-Competence Broth (Nippongene) was added thereto, incubated at 37°C for 1 hour, and then the E. coli was plated on the LBA agar medium and incubated overnight at 37°C to obtain an E. coli transformant. The transformant was cultured overnight in 3 ml of the LBA medium, and from the cell fractions, plasmid DNA was prepared using the QIAprep Spin Plasmid Kit (QIAGEN).

The nucleotide sequence of the cDNA coding region in the plasmid was determined using the Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer) by the DNA Sequencer 373A (Perkin-Elmer). As the sequencing primer, M13 Primer M4 (Takara Shuzo) and M13 Primer RV (Takara Shuzo) were used, and the sequence was determined by confirming the nucleotide sequence in both directions. The plasmid that contains the gene encoding the humanized antibody L chain V region and that has a BglII recognition sequence and the Kozak sequence at the 5'-end, and a SphI recognition sequence at the 3'-end was designated as hATR5Lva/CVIDEC. The nucleotide sequence (including the corresponding amino acid sequence) of the

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with ethanol, they were dissolved in TE.

The KpnI-PstI DNA fragment prepared as above encoding the FR3 of versions "b" or "c" and the hATR5Lva/CVIDEC vector in which the FR3 was removed by
5 digesting with KpnI and PstI were ligated using the DNA ligation kit ver.2 (Takara Shuzo) by reacting at 16°C for 1 hour according to the instructions attached to the kit.

The ligation mixture was added to 100 µl of E. coli JM109 competent cells (Nippongene) and was incubated for
10 30 minutes on ice and for 1 minute at 42°C. Then, 300 µl of the Hi-Competence Broth (Nippongene) was added thereto, incubated at 37°C for 1 hour, and then the E. coli was plated on the LBA agar medium and incubated overnight at 37°C to obtain an E. coli transformant. The
15 transformant was cultured overnight in 3 ml of the LBA medium, and from the cell fractions, plasmid DNA was prepared using the QIAprep Spin Plasmid Kit (QIAGEN).

The nucleotide sequence of the cDNA coding region in the plasmid was determined using the Dye Terminator Cycle
20 Sequencing FS Ready Reaction Kit (Perkin-Elmer) by the DNA Sequencer 373A (Perkin-Elmer). As the sequencing primer, M13 Primer M4 (Takara Shuzo) and M13 Primer RV (Takara Shuzo) were used, and the sequence was determined by confirming the nucleotide sequence in both directions.

The plasmids that contain the gene encoding version "b" or version "c" in which the FR3 of humanized antibody L chain version "a" was replaced was designated as hATR5Lvb/CVIDEC or hATR5Lvc/CVIDEC, respectively. The
25 nucleotide sequence and the corresponding amino acid sequence of the humanized L chain version "b" contained in plasmid hATR5Lvb/CVIDEC and the amino acid sequence of version "b" are shown in SEQ ID NO: 87 and 88. The
30 nucleotide sequence and the corresponding amino acid sequence of the humanized L chain version "c" contained in plasmid hATR5Lvc/CVIDEC and the amino acid sequence of
35 version "c" are shown in SEQ ID NO: 89 and 90.

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digesting with AflIII and SpeI were ligated using the DNA ligation kit ver.2 (Takara Shuzo) by reacting at 16°C for 1 hour according to the instructions attached to the kit.

The ligation mixture was added to 100 µl of E. coli JM109 competent cells (Nippongene) and was incubated for 30 minutes on ice and for 1 minute at 42°C. Then, 300 µl of the Hi-Competence Broth (Nippongene) was added thereto, incubated at 37°C for 1 hour, and then the E. coli was plated on the LBA agar medium and incubated overnight at 37°C to obtain an E. coli transformant. The transformant was cultured overnight at 37°C in 4 ml of the LBA medium, and from the cell fractions, plasmid DNA was prepared using the QIAprep Spin Plasmid Kit (QIAGEN).

The nucleotide sequence of the cDNA coding region in the plasmid was determined using the Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer) by the DNA Sequencer 373A (Perkin-Elmer). As the sequencing primer, M13 Primer M4 (Takara Shuzo) and M13 Primer RV (Takara Shuzo) were used, and the sequence was determined by confirming the nucleotide sequence in both directions.

The plasmids that contain the gene encoding version "b1" or "b2" in which the FR2 of humanized antibody L chain version "b" was replaced was designated as hATR5Lvbl/CVIDEC and hATR5Lv2/CVIDEC, respectively. The nucleotide sequence and the corresponding amino acid sequence of the humanized L chain version "b1" contained in plasmid hATR5Lvbl/CVIDEC and the amino acid sequence of version "b1" are shown in SEQ ID NO: 95 and 96. The nucleotide sequence and the corresponding amino acid sequence of the humanized L chain version "b2" contained in plasmid hATR5Lv2/CVIDEC and the amino acid sequence of version "b2" are shown in SEQ ID NO: 97 and 98.

(3) Construction of the expression vector of humanized antibody

(i) Combination of humanized H chain and chimeric L chain

The plasmid hATR5Hva/CVIDEC containing a H chain V region was digested with NheI and SalI, and a cDNA fragment of the humanized H chain V region was recovered and introduced to chATR5/N5KG4P (SalI/NheI) prepared by
 5 digesting chATR5/N5KG4P, a chATR-5 antibody expression plasmid vector, with NheI and SalI. The plasmid thus generated was designated as hHva-chLv/N5KG4P.

The plasmid hATR5Hvb/CVIDEC containing a H chain V region was digested with NheI and SalI, and a cDNA
 10 fragment of the humanized H chain V region was recovered and introduced to chATR5/N5KG4P (SalI/NheI) prepared by digesting chATR5/N5KG4P, a chATR-5 antibody expression plasmid vector, with NheI and SalI. The plasmid thus generated was designated as hHvb-chLv/N5KG4P.

The plasmids hATR5Hvc/CVIDEC, hATR5Hvd/CVIDEC, and hATR5Hve/CVIDEC containing a H chain V region were digested with NheI and SalI, and cDNA fragments of the humanized H chain V region were recovered and introduced to chATR5/N5KG4P (SalI/NheI) prepared by digesting
 15 chATR5/N5KG4P, a chATR-5 antibody expression plasmid vector, with NheI and SalI. The plasmids thus generated were designated as hHvc-chLv/N5KG4P, hHvd-chLv/N5KG4P, and hHve-chLv/N5KG4P.

The plasmids hATR5Hvf/CVIDEC and hATR5Hvh/CVIDEC containing a H chain V region were digested with NheI and SalI, and cDNA fragments of the humanized H chain V region were recovered and introduced to chATR5/N5KG4P (SalI/NheI) prepared by digesting chATR5/N5KG4P, a chATR-
 25 5 antibody expression plasmid vector, with NheI and SalI. The plasmids thus generated were designated as hHvf-chLv/N5KG4P and hHvh-chLv/N5KG4P.

The plasmids hATR5Hvi/CVIDEC and hATR5Hvj/CVIDEC containing a H chain V region were digested with NheI and SalI, and cDNA fragments of the humanized H chain V
 35 region were recovered and introduced to chATR5/N5KG4P (SalI/NheI) prepared by digesting chATR5/N5KG4P, a chATR-5 antibody expression plasmid vector, with NheI and SalI.

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thereto, incubated at 37°C for 1 hour, and then the E. coli was plated on the LBA agar medium and incubated overnight at 37°C to obtain an E. coli transformant.

The transformant was cultured overnight at 37°C in 250 ml or 500 ml of the LBA medium, and from the cell fractions, plasmid DNA was prepared using the Plasmid Maxi Kit (QIAGEN). The plasmids in which a gene encoding the chimeric H chain and humanized L chain was introduced were designated as chHv-hLva/N5KG4P, chHv-hLvb/N5KG4P, chHv-hLvc/N5KG4P, chHv-hLvb1/N5KG4P, and chHv-hLvb2/N5KG4P.

(iii) Combination of humanized H chain and humanized L chain

The plasmid hATR5Hva/CVIDEC containing a H chain V region was digested with NheI and SalI, and a cDNA fragment of the humanized H chain V region was recovered and introduced to hLva/N5KG4P (SalI/NheI) prepared by digesting plasmid chHv-hLva/N5KG4P containing the cDNA sequence of humanized ATR-5 antibody L chain version "a" with NheI and SalI. The plasmid thus generated was designated as hHva-hLva/N5KG4P.

The plasmids hATR5Hvb/CVIDEC and hATR5Hvc/CVIDEC containing a H chain V region were digested with NheI and SalI, and cDNA fragments of the humanized H chain V region were recovered and introduced to hLva/N5KG4P (SalI/NheI) prepared by digesting plasmid chHv-hLva/N5KG4P containing the cDNA sequence of humanized ATR-5 antibody L chain version "a" with NheI and SalI. The plasmids thus generated were designated as hHvb-hLva/N5KG4P and hHvc-hLva/N5KG4P.

The plasmids hATR5Hvb/CVIDEC, hATR5Hvd/CVIDEC, and hATR5Hve/CVIDEC containing a H chain V region were digested with NheI and SalI, and cDNA fragments of the humanized H chain V region were recovered and introduced to hLvb/N5KG4P (SalI/NheI) prepared by digesting plasmid chHv-hLvb/N5KG4P containing the cDNA sequence of humanized ATR-5 antibody L chain version "b" with NheI

and SalI. The plasmids thus generated were designated as hHvb-hLvb/N5KG4P, hHvd-hLvb/N5KG4P, and hHve-hLvb/N5KG4P.

The plasmids hATR5Hvf/CVIDEC, hATR5Hvg/CVIDEC, and hATR5Hvh/CVIDEC containing a H chain V region were
5 digested with NheI and SalI, and cDNA fragments of the humanized H chain V region were recovered and introduced to hLvb/N5KG4P (SalI/NheI) prepared by digesting plasmid chHv-hLvb/N5KG4P containing the cDNA sequence of
humanized ATR-5 antibody L chain version "b" with NheI
10 and SalI. The plasmids thus generated were designated as hHvf-hLvb/N5KG4P, hHvg-hLvb/N5KG4P, and hHvh-hLvb/N5KG4P.

The plasmids hATR5Hvi/CVIDEC and hATR5Hvj/CVIDEC containing a H chain V region were digested with NheI and SalI, and cDNA fragments of the humanized H chain V
15 region were recovered and introduced to hLvb/N5KG4P (SalI/NheI) prepared by digesting plasmid chHv-hLvb/N5KG4P containing the cDNA sequence of humanized ATR-5 antibody L chain version "b" with NheI and SalI.
The plasmids thus generated were designated as hHvi-hLvb/N5KG4P and hHvj-hLvb/N5KG4P.
20

The plasmids hATR5Hvb1/CVIDEC and hATR5Hvd1/CVIDEC containing a H chain V region were digested with NheI and SalI, and cDNA fragments of the humanized H chain V
region were recovered and introduced to hLvb/N5KG4P
25 (SalI/NheI) prepared by digesting plasmid chHv-hLvb/N5KG4P containing the cDNA sequence of humanized ATR-5 antibody L chain version "b" with NheI and SalI.
The plasmids thus generated were designated as hHvb1-hLvb/N5KG4P and hHvd1-hLvb/N5KG4P.

The plasmids hATR5Hvb3/CVIDEC and hATR5Hvd3/CVIDEC containing a H chain V region were digested with NheI and SalI, and cDNA fragments of the humanized H chain V
region were recovered and introduced to hLvb/N5KG4P
30 (SalI/NheI) prepared by digesting plasmid chHv-hLvb/N5KG4P containing the cDNA sequence of humanized ATR-5 antibody L chain version "b" with NheI and SalI.
The plasmids thus generated were designated as hHvb3-

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dish or 15 cm culture dish in a 5% CO₂ incubator. After culturing for 24 hours, the culture supernatant was aspirated off, and then a serum-free medium HBCHO (Irvine Scientific) was added. After further culturing for 72 hours or 96 hours, the culture supernatant was collected and centrifuged to remove cell debris.

(5) Purification of antibody

From the culture supernatant of the COS-7 cells, the antibody was purified using the AffiGel Protein A MAPSII kit (Bio Rad) or the rProtein A Sepharose Fast Flow (Pharmacia Biotech). Purification using the AffiGel Protein A MAPSII kit was carried out according to the instructions attached to the kit. Purification using the rProtein A Sepharose Fast Flow was carried out as follows:

One ml of rProtein A Sepharose Fast Flow was filled into a column and the column was equilibrated by 10 volumes of TBS. The culture supernatant of COS-7 cells was applied to the equilibrated column, which was then washed with 10 volumes of TBS. The adsorbed antibody fraction was eluted by 13.5 ml of 2.5 mM HCl (pH 3.0). The eluate was neutralized by adding 1.5 ml of 1 M Tris-HCl (pH 8.0).

By performing ultrafiltration two or three times for the purified antibody fraction using the Centriprep 30 or 100 (amicon), the solvent was replaced to TBS, and was finally concentrated to about 1.5 ml.

Reference Example 6. Antibody quantitation and activity evaluation

(1) Measurement of antibody concentration by ELISA
ELISA plates for measurement of antibody concentration were prepared as follows: Each well of a 96-well ELISA plate (Maxisorp, NUNC) was immobilized by 100 µl of goat anti-human IgGγ antibody (BIO SOURCE) prepared to a concentration of 1 µg/ml in the immobilization buffer (0.1 M NaHCO₃, 0.02% NaN₃, pH 9.6)

(hereinafter referred to as CB). After blocking with 200 μ l of the dilution buffer (50 mM Tris-HCl, 1 mM MgCl₂, 0.1 M NaCl, 0.05% Tween 20, 0.02% NaN₃, 1% bovine serum albumin (BSA), pH 8.1) (hereinafter referred to as DB),
 5 the culture supernatant of the COS-7 cells in which antibody was expressed or purified antibody were serially diluted with DB, and then added to each well.

After incubating at room temperature for 1 hour followed by washing with the Dulbecco PBS containing
 10 0.05% Tween 20 (hereinafter referred to as RB), 100 μ l of alkaline phosphatase-conjugated goat anti-human IgG γ antibody (Biosource) which was diluted 1000-fold with DB was added. After incubating at room temperature for 1 hour followed by washing with the RB, Sigma104
 15 (p-nitrophenyl phosphate, SIGMA) dissolved in the substrate buffer (50 mM NaHCO₃, 10 mM MgCl₂, pH 9.8) to 1 mg/ml was added, and then the absorbance at 405/655 nm was measured using the Microplate Reader (Bio Rad). As the standard for the measurement of concentration, IgG4k
 20 (Binding Site) was used.

(2) Measurement of the activity of binding to the antigen

Cell ELISA plates for measurement of antigen binding were prepared as follows. Cells used were human bladder
 25 carcinoma cells J82 (ATCC HTB-1). To 60 wells of a 96-well cell culture plate, 1×10^5 J82 cells were inoculated. This was cultured (RPMI1640 medium containing 10% fetal bovine serum (GIBCO)) for one day in a CO₂ incubator to allow the cells to be attached
 30 thereto. After discarding the culture liquid, each well was washed twice with 300 μ l PBS. 100 μ l of PBS containing 4% paraformaldehyde (hereinafter referred to as PFA/PBS) was added to each well, and placed on ice for 10 minutes to immobilize the cells.

35 PFA/PBS was discarded, and each well was washed

twice with 300 μ l of PBS, and then blocked with 250 μ l of DB. The culture supernatant or purified antibody was serially diluted with DB, 100 μ l of which was added to each well. After incubating at room temperature for 2 hours followed by washing with RB, 100 μ l of alkaline phosphatase-conjugated goat anti-human IgG γ antibody (BioSource) diluted 1000-fold with DB was added. After incubating for 1 hour followed by washing with RB, the substrate solution was added, and then absorbance at 405/655 nm was measured using the Microplate Reader (Bio-Rad).

(3) Measurement of neutralizing activity

The neutralizing activity of mouse antibody, chimeric antibody, and humanized antibody was measured with the inhibiting activity against the Factor Xa-production activity by human placenta-derived thromboplastin, Thromborel S (Boehringer AG), as an index. Thus, 60 μ l of the buffer (TBS containing 5 mM CaCl₂ and 0.1% BSA) was added to 10 μ l of 1.25 mg/ml Thromborel S and 10 μ l of appropriately diluted antibody, which was then incubated in a 96-well plate at room temperature for 1 hour. Ten μ l each of 3.245 μ g/ml human Factor X (Celsus Laboratories) and 82.5 ng/ml human Factor VIIa (Enzyme Research) were added thereto, and then were incubated at room temperature for 1 hour.

Ten μ l of 0.5 M EDTA was added to stop the reaction, to which 50 μ l of the chromogenic substrate solution was added and the absorbance at 405/655 nm was determined using the Microplate Reader (Bio Rad). After reacting at room temperature for 1 hour, the absorbance at 405/655 nm was determined again. The neutralizing activity may be determined by calculating the residual activity (%) from each change in absorbance with the hourly absorbance change at no antibody addition as a 100% activity.

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The chromogenic substrate solution was prepared by dissolving the Testzyme chromogenic substrate S-2222 (Chromogenix) according to the attached instructions, diluting 2-fold with purified water and mixing with a polybrene solution (0.6 mg/ml hexadimethylene bromide, SIGMA) at 1:1.

(4) Evaluation of activity

(i) Combination of the humanized H chain version "a" and a chimeric L chain

10 An antibody (a-ch) which is the humanized H chain version "a" combined with a chimeric L chain was generated, and was tested for the binding activity to the antigen by the cell ELISA. The amount bound to the antigen was found to be decreased at the high
15 concentration. The neutralizing activity against the antigen by FXa production-inhibition was weak as compared that of to the positive control chimeric antibody (ch-ch). Therefore, it was decided to perform the version-up of the humanized H chain by FR-shuffling. The chimeric
20 antibody used herein was the one that was expressed in COS-7 cells, purified, and evaluated.

(ii) Combination of the humanized L chain version "a" and a chimeric H chain

25 An antibody (ch-a) which is the humanized L chain version "a" combined with a chimeric H chain was generated, and was tested for the binding activity to the antigen by the cell ELISA. It was found to have the binding activity equal to or higher than that of the chimeric antibody. On the other hand, the neutralizing
30 activity against the antigen was weak as compared to that of the positive control chimeric antibody. Therefore, it was decided to perform the version-up of the humanized L chain by FR-shuffling. The chimeric antibody used herein was the one that was expressed in COS-7 cells, purified,
35 and evaluated.

(iii) Combination of the humanized H chain version "a" and the humanized L chain version "a"

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An antibody (a-a) which is the humanized H chain version "a" combined with the humanized L chain version "a" was generated, and was tested for the binding activity to the antigen by the cell ELISA. The amount
 5 bound to the antigen was found to be decreased in the high concentration side. The neutralizing activity against the antigen by FXa production-inhibition was weak as compared to that of the positive control chimeric antibody. Therefore, it was decided to perform the
 10 version-up of the humanized H chain and L chain by FR-shuffling. The chimeric antibody used herein was the one that was expressed in COS-7 cells, purified, and evaluated.

(iv) Combination of the humanized H chain versions
 15 "b", "c", and "d", and a chimeric L chain Antibodies ("b-ch", "c-ch", and "d-ch", respectively) which are the humanized H chain subjected to version-up by FR-shuffling combined with a chimeric L chain were generated, and were tested for the binding
 20 activity to the antigen by the cell ELISA. "d-ch" exhibited a binding activity equal to that of the chimeric antibody, and "b-ch" and "c-ch" exhibited a slightly lower binding activity. On the other hand, the neutralizing activity against the antigen as compared to
 25 the that of positive control chimeric antibody was almost equal in "b-ch", and slightly weak in "d-ch". In version "c-ch", it was significantly weaker than that of the chimeric antibody. Therefore, the humanized H chain versions "b" and "d" were considered the ones of the
 30 humanized H chain to exhibit a high activity.

(v) Combination of the humanized H chain version "b" and the humanized L chain version "a"

An antibody (b-a) which is the humanized H chain version "b" subjected to version-up by FR-shuffling
 35 combined with the humanized L chain version "a" was generated, and was tested for the binding activity to the antigen by the cell ELISA. The amount bound to the

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antigen was found to be decreased at the high concentration. On the other hand, the neutralizing activity against the antigen was significantly weak as compared to that of the positive control chimeric antibody. Therefore, "b-a" and "a-a" were the ones that exhibit a high activity. The chimeric antibody used herein was the one that was expressed in COS-7 cells, purified, and evaluated.

(vi) Combination of the humanized L chain versions "b" and "c", and a chimeric H chain

Antibodies ("ch-b" and "ch-c", respectively) which are the humanized L chain versions "b" and "c" combined with a chimeric H chain were generated, and both of them were found to have the binding activity to the antigen and the neutralizing activity against the antigen equal to the chimeric antibody. Therefore, versions "b" and "c" were chosen as a candidate for a humanized antibody L chain. Mouse antibody-derived version "b" which is one amino acid fewer in the amino acid residue number is considered to be superior to version "c" in terms of antigenicity. The chimeric antibody used herein was the one that was expressed in CHO cells DG44, purified, and evaluated. In the evaluation hereinafter the antibody was used as the positive control.

(vii) Combination of the humanized H chain version "b" and the humanized L chain versions "b" and "c"

Antibodies ("b-b" and "b-c", respectively) which are the humanized H chain version "b" combined with the humanized L chain versions "b" and "c" were generated, and tested for the binding activity to the antigen and the neutralizing activity against the antigen. Both of them had a slightly lower activity than that of the chimeric antibody in both the binding activity and the neutralizing activity.

(viii) Combination of the humanized H chain versions "b" and "d", and the humanized L chain

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antibody expressed was very little. For versions "f" and "h", antibodies combined with the chimeric L chain were generated, but were not expressed. "g-b" reached saturation at a low concentration, and exhibited a binding activity weaker than that of the chimeric antibody. The neutralizing activity against the antigen of "g-b" was significantly weak as compared to that of the chimeric antibody.

(xi) Combination of the humanized H chain versions "b1" and "d1", and the humanized L chain version "b"

Antibodies ("b1-b" and "d1-b", respectively) which are the humanized H chain versions "b1" and "d1" combined with the humanized L chain version "b" were generated. Almost no antibody was expressed in any of them. For these, antibodies combined with a chimeric L chain were generated, but were not expressed.

(xii) Combination of the humanized H chain versions "b3" and "d3", and the humanized L chain version "b"

Antibodies ("b3-b" and "d3-b", respectively) which are the humanized H chain versions "b3" and "d3" combined with the humanized L chain version "b" were generated. The binding activity to the antigen of "d3-b" was slightly lower than that of the chimeric antibody, and that of "b3-b" was much lower. The neutralizing activity against the antigen of "b3-b" was higher than that of "b-b", but was lower than that of the chimeric antibody, and "d3-b" and "b-b" remained equal in activity.

(xiii) Combination of the humanized H chain versions "i" and "j", and a chimeric L chain and the humanized L chain version "b"

Antibodies ("i-ch" and "j-ch", respectively) which are the humanized H chain versions "i" and "j" combined with a chimeric L chain, and antibodies ("i-b" and "j-b", respectively) combined with the humanized L chain version "b" were generated, and were tested for the binding

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are the humanized H chain version "i" combined with the humanized L chain version "b1" or "b2" were generated, and were tested for the binding activity to the antigen and the neutralizing activity against the antigen. The binding activity of "i-b2" was almost equal to that of the chimeric antibody, and that of "i-b1" was slightly lower than that of chimeric antibody. The neutralizing activity of "i-b1" and "i-b2" was higher than that of the chimeric antibody and "i-b", which was in a decreasing order of "i-b2" > "i-b1".

Reference Example 7. Preparation of CHO cell-producing humanized antibody and the evaluation of its activity

(1) Establishment of a cell line that stably produces CHO

In order to establish cell lines that stably produce a humanized antibody (b-b, i-b, and i-b2), an antibody expression gene vector was introduced into CHO cells (DG44) acclimated to a serum-free medium.

Plasmid DNA, hHvb-hLvb/N5KG4P, hHvi-hLvb/N5KG4P, and hHvi-hLvb2/N5KG4P were digested with the restriction enzyme SspI (Takara Shuzo) and linearized, which was extracted with phenol and chloroform, and purified by ethanol precipitation. The linearized expression gene vector was introduced into the DG44 cells using the electroporation instrument (Gene Pulser; Bio Rad). The DG44 cells were suspended in PBS at a cell concentration of 1×10^7 cells/ml, and to about 0.8 ml of this suspension 10 or 50 μ g of the DNA was added, which was subjected to pulses of 1,500 V and 25 μ F capacity.

After 10 minutes of the recovery period at room temperature, the treated cells were suspended in a CHO-S-SFMII medium (GIBCO) containing hypoxanthine/thymidine (GIBCO) (hereinafter referred to as HT), which was inoculated on two 96-well plates (Falcon) at 100 μ l/well,

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and cultured in a CO₂ incubator. Eight to nine hours after the start of culturing, 100 µl/well of the CHO-S-SFMII medium containing HT and 1 mg/ml GENETICIN (GIBCO) was added to change to 500 µg/ml of the GENETICIN selection medium, and the cells into which the antibody gene had been introduced were selected. The medium was changed with a fresh one once every 3-4 days with 1/2 the volume. At a time point about 2 weeks after changing to the selection medium, an aliquot of the culture supernatant was recovered from the well in which a favorable cell growth was observed 4-5 days later. The concentration of antibody expressed in the culture supernatant was measured by the ELISA described above for measuring antibody concentration, and cells having a high production yield of antibody were selected.

(2) Large scale purification of humanized antibody
After the DG44 cell lines selected as above that produce the humanized antibody ("b-b", "i-b", and "i-b2") were cultured for a few days in a 500 ml/bottle of the CHO-S-SFMII medium using a 2 L roller bottle (CORNING), the culture medium was harvested and a fresh CHO-S-SFMII medium was added and cultured again. The culture medium was centrifuged to remove the cell debris, and filtered with a 0.22 µm or 0.45 µm filter. By repeating this, a total of about 2 L each of the culture supernatant was obtained. From the culture supernatant obtained, antibody was purified by the ConSep LC100 system (Millipore) connected to the Protein A affinity column (Poros).

(3) Measurement of antibody concentration by ELISA
ELISA plates for measurement of antibody concentration were prepared as follows: Each well of a 96-well ELISA plate (Maxisorp, NUNC) was immobilized with 100 µl of goat anti-human IgGγ antibody (BioSource) prepared to a concentration of 1 µg/ml with CB. After

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blocking with 200 μ l of DB, the culture supernatant of the CHO cells in which antibody had been expressed or the purified antibody was serially diluted with DB, and added to each well.

5 After incubating at room temperature for 1 hour and washing with RB, 100 μ l of alkaline phosphatase-conjugated goat anti-human IgG antibody (BioSource) diluted 1000-fold with DB was added. After incubating at room temperature for 1 hour and washing
10 with RB, 100 μ l of the substrate solution was added, and then the absorbance at 405/655 nm was measured using the Microplate Reader (Bio Rad). As the standard for the measurement of concentration, human IgG4k (The Binding Site) was used.

15 (4) Measurement of activity of binding to the antigen

Cell ELISA plates for measurement of antigen binding were prepared as follows. Cells used were human bladder carcinoma cells J82 (ATCC HTB-1), which were inoculated
20 onto a 96-well cell culture plate at a cell count of 1×10^5 cells. This was cultured (RPMI1640 medium containing 10% fetal bovine serum (GIBCO)) for one day in a CO₂ incubator to allow the cells to be attached thereto. After discarding the culture liquid, each well was washed
25 twice with PBS. 100 μ l of PFA/PBS was added to each well, and placed on ice for 10 minutes to immobilize the cells.

PFA/PBS was discarded, and each well was washed twice with 300 μ l of PBS and then blocked with 250 μ l of
30 DB. Based on the above result of measurement, the purified antibody was serially diluted with DB starting at 10 μ g/ml by a factor of 2, 100 μ l of which was added to each well. After incubating at room temperature for 2 hours and washing with RB, 100 μ l of alkaline

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phosphatase-conjugated goat anti-human IgG γ antibody (BioSource) diluted 1000-fold with DB was added. After incubating at room temperature for 1 hour and washing with RB, 100 μ l of the substrate solution was added, and then absorbance at 405/655 nm was measured using the Microplate Reader (Bio-Rad).

(5) Measurement of neutralizing activity against TF (Factor inhibiting activity against the FXa production)

The Factor Xa production-inhibiting activity of humanized antibody was measured with the inhibiting activity against the Factor Xa production activity by the human placenta-derived thromboplastin, Thromborel S (Boehringer AG), as an index. Thus, 60 μ l of the buffer (TBS containing 5 mM CaCl₂ and 0.1% BSA) was added to 10 μ l of 5 mg/ml Thromborel S and 10 μ l of the antibody, which was then incubated in a 96-well plate at room temperature for 1 hour. The antibody was serially diluted with the buffer starting at 200 μ g/ml by a factor of 5.

Ten μ l each of 3.245 μ g/ml human Factor X (Celsus Laboratories) and 82.5 ng/ml human Factor VIIa (Enzyme Research) were added thereto, and were further incubated at room temperature for 45 minutes. Ten μ l of 0.5 M EDTA was added to stop the reaction. Fifty μ l of the chromogenic substrate solution was added thereto and the absorbance at 405/655 nm was determined by the Microplate Reader (Bio Rad). After reacting at room temperature for 30 minutes, the absorbance at 405/655 nm was measured again. The residual activity (%) was determined from each change in absorbance with the absorbance change for 30 minutes at no antibody addition as a 100% activity.

The chromogenic substrate solution was prepared by dissolving the Testzyme chromogenic substrate S-2222 (Chromogenix) according to the attached instructions, and

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S concentration was plotted on the abscissa, and the coagulation time on the ordinate on a log-log paper, which was rendered a standard curve.

(8) Activity evaluation

5 All humanized antibodies, "b-b", "i-b", and "i-b2" had an activity equal to or greater than that of the chimeric antibody (Figure 1). For inhibiting activity against FXa production, inhibiting activity FX-binding, and inhibiting activity against plasma coagulation as
10 well, the humanized antibodies, "b-b", "i-b", and "i-b2" had an activity equal to or greater than that of the chimeric antibody, and the activity was of a decreasing order "i-b2" > "i-b" > "b-b" (Figures 2, 3, and 4).

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CLAIMS

1. An experimental animal having implanted therein an animal cell to which the gene encoding human tissue factor (TF) or part thereof has been inserted and which is capable of expressing said gene, said animal being a non-human animal in which a hypercoagulable state persists for a long period of time.

2. The animal according to claim 1 wherein said part of human tissue factor lacks the intracellular region.

3. The animal according to claim 1 or 2 wherein said animal cell is a mammalian cell.

4. The animal according to claim 3 wherein said mammalian cell is a human myeloma cell.

5. The animal according to any one of claims 1 to 4 wherein said animal is a mouse.

6. The animal according to any of claims 1 to 5 wherein said hypercoagulable state is indicated by at least one of the phenomena comprising an increase in the plasma concentration of human tissue factor, a decrease in platelets, a decrease in fibrinogen, an increase in the concentration of soluble fibrin monomer complex, and an increase in the concentration of thrombin-antithrombin III complex.

7. A method of generating the animal according to any one of claims 1 to 6, wherein an animal cell to which the gene encoding human tissue factor (TF) or part thereof has been inserted and which is capable of expressing said gene is implanted to non-human animals and then an animal having a persistent hypercoagulable state is selected.

8. A method of screening an anti-thrombotic agent which method comprises using the animal according to any one of claims 1 to 6.

9. A preventive or therapeutic agent for diseases having a persistent hypercoagulable state, said agent comprising an antibody to human tissue factor (human TF).

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10. The preventive or therapeutic agent according to claim 9 wherein said antibody is a polyclonal antibody.

5 11. The preventive or therapeutic agent according to claim 9 wherein said antibody is a monoclonal antibody.

12. The preventive or therapeutic agent according to claim 9 or 11 wherein said antibody is a recombinant antibody.

10 13. The preventive or therapeutic agent according to claim 9 or 12 wherein said antibody is an altered antibody.

14. The preventive or therapeutic agent according to claim 9, 12, or 13 wherein said altered antibody is a chimeric antibody or a humanized antibody.

15 15. The preventive or therapeutic agent according to claim 14 wherein said humanized antibody is a humanized antibody of version b-b, i-b, or i-b2.

20 16. The preventive or therapeutic agent according to claim 9 or any one of claims 12-15 wherein said antibody is a modified antibody.

25 17. The preventive or therapeutic agent according to claim 16 wherein said modified antibody is an antibody fragment Fab, F(ab')₂, or Fv, or a single chain Fv (scFv).

18. A preventive or therapeutic agent for a hypercoagulable state resulting from infections, said agent comprising an antibody against human tissue factor (human TF).

30 19. The preventive or therapeutic agent according to claim 18 wherein said antibody is a polyclonal antibody.

35 20. The preventive or therapeutic agent according to claim 18 wherein said antibody is a monoclonal antibody.

21. The preventive or therapeutic agent according to claim 18 or 20 wherein said antibody is a recombinant

antibody.

22. The preventive or therapeutic agent according to claim 18 or 21 wherein said antibody is an altered antibody.

5 23. The preventive or therapeutic agent according to claim 18, 21, or 22 wherein said altered antibody is a chimeric antibody or a humanized antibody.

24. The preventive or therapeutic agent according to claim 23 wherein said humanized antibody is a
10 humanized antibody of version b-b, i-b, or i-b2.

25. The preventive or therapeutic agent according to claim 18 or any one of claims 21-24 wherein said antibody is a modified antibody.

26. The preventive or therapeutic agent according to claim 25 wherein said modified antibody is an antibody
15 fragment Fab, F(ab')₂, or Fv, or a single chain Fv (scFv).

27. A preventive or therapeutic agent for venous thrombosis, said agent comprising an antibody to human
20 tissue factor (human TF).

28. The preventive or therapeutic agent according to claim 27 wherein said antibody is a polyclonal antibody.

29. The preventive or therapeutic agent according to claim 27 wherein said antibody is a monoclonal
25 antibody.

30. The preventive or therapeutic agent according to claim 27 or 29 wherein said antibody is a recombinant antibody.

31. The preventive or therapeutic agent according to claim 27 or 30 wherein said antibody is an altered
30 antibody.

32. The preventive or therapeutic agent according to claim 27, 30, or 31 wherein said altered antibody is a
35 chimeric antibody or a humanized antibody.

33. The preventive or therapeutic agent according to claim 32 wherein said humanized antibody is a

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humanized antibody of version b-b, i-b, or i-b2.

34. The preventive or therapeutic agent according to claim 27 or any one of claims 30-33 wherein said antibody is a modified antibody.

5 35. The preventive or therapeutic agent according to claim 34 wherein said modified antibody is an antibody fragment Fab, F(ab')₂, or Fv, or a single chain Fv (scFv).

10 36. A preventive or therapeutic agent for arterial thrombosis, said agent comprising an antibody to human tissue factor (human TF).

37. The preventive or therapeutic agent according to claim 36 wherein said antibody is a polyclonal antibody.

15 38. The preventive or therapeutic agent according to claim 36 wherein said antibody is a monoclonal antibody.

20 39. The preventive or therapeutic agent according to claim 36 or 38 wherein said antibody is a recombinant antibody.

40. The preventive or therapeutic agent according to claim 36 or 39 wherein said antibody is an altered antibody.

25 41. The preventive or therapeutic agent according to claim 36, 39, or 40 wherein said altered antibody is a chimeric antibody or a humanized antibody.

42. The preventive or therapeutic agent according to claim 41 wherein said humanized antibody is a humanized antibody of version b-b, i-b, or i-b2.

30 43. The preventive or therapeutic agent according to claim 36 or any one of claims 39-42 wherein said antibody is a modified antibody.

35 44. The preventive or therapeutic agent according to claim 43 wherein said modified antibody is an antibody fragment Fab, F(ab')₂, or Fv, or a single chain Fv (scFv).

45. A preventive or therapeutic agent for diseases

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ABSTRACT

5 This invention provides an animal having a
persistent hypercoagulable state by implanting a cell,
for example a tumor cell, in which the gene of human
tissue factor is implanted to an experimental animal such
as a mouse and then by growing said cell, thereby
persistently supplying human tissue factor to said
experimental animal. This animal model is useful for
10 research and development of therapeutic agents for
diseases having a persistent hypercoagulable state.

The present invention also provides preventive or
therapeutic agents for diseases having a persistent
hypercoagulable state, a hypercoagulable state resulting
15 from infections, venous thrombosis, arterial thrombosis,
and diseases resulting from the hypertrophy of vascular
media, said agent comprising an antibody against human
tissue factor (human TF) as an active ingredient.

Fig.1

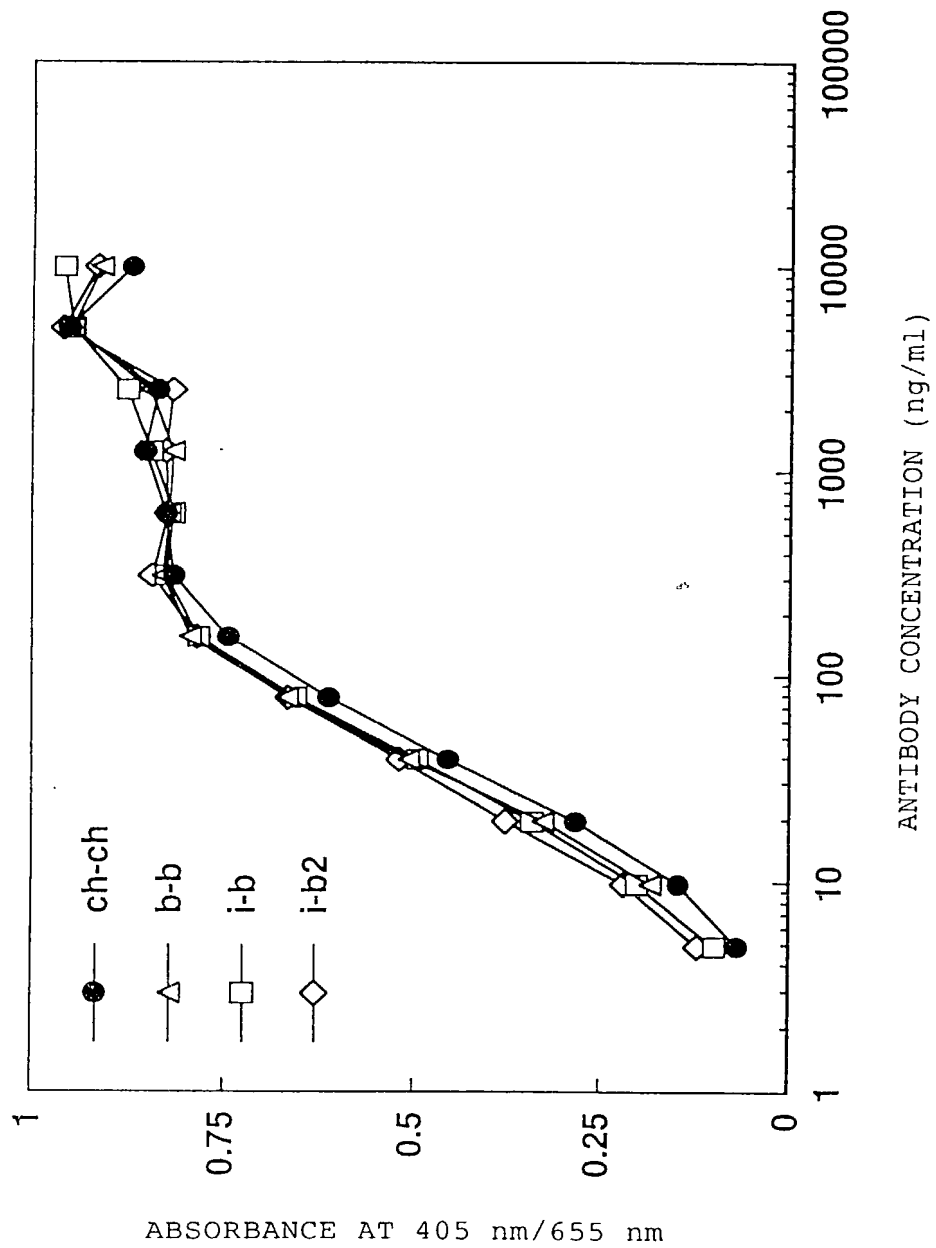
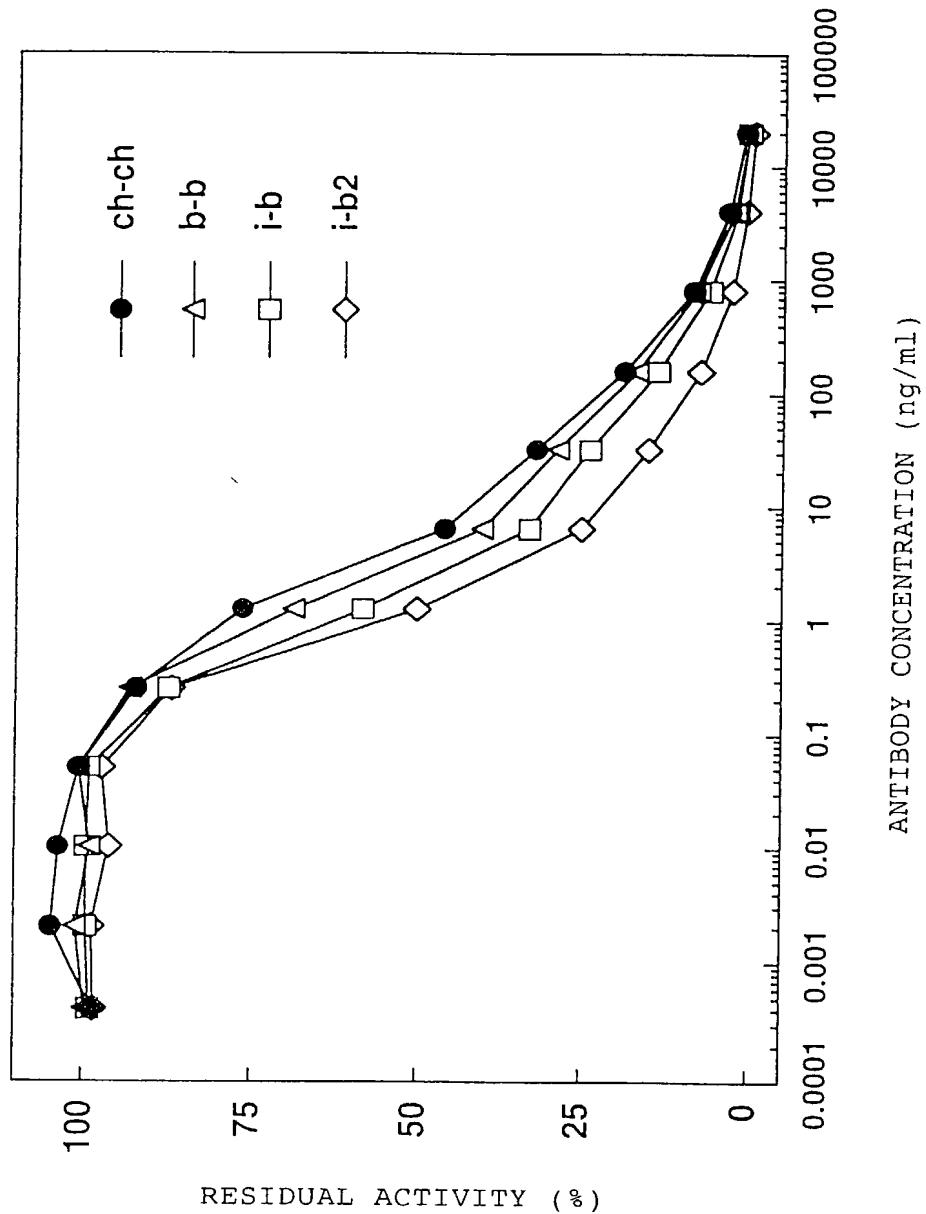


Fig.2

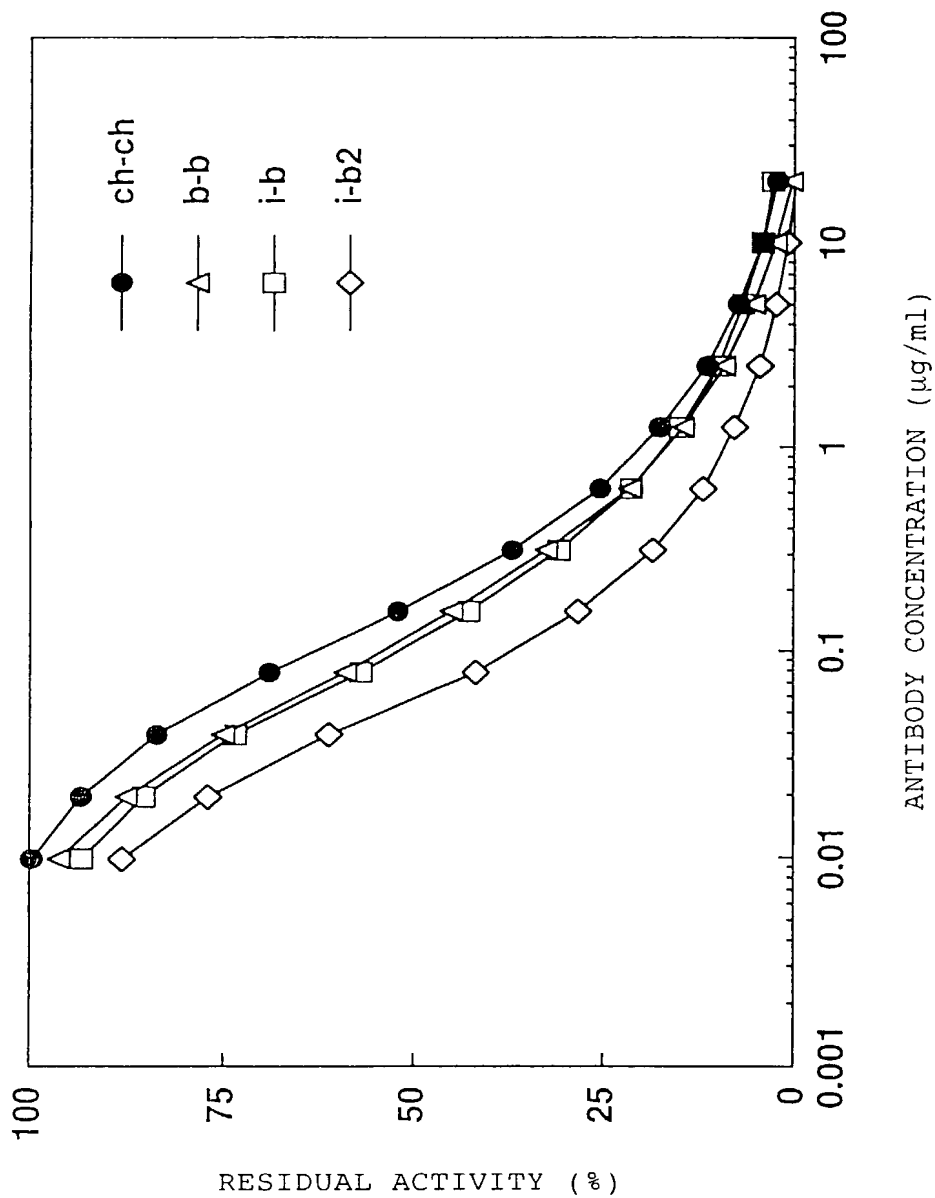


Title: PREVENTION AND TREATMENT
OF BLOOD COAGULATION-RELATED
DISEASES

Inventor(s): Hiroyuki SAITO et al.
DOCKET NO.: 053466-0325

10/089501

Fig. 3



Title: PREVENTION AND TREATMENT
OF BLOOD COAGULATION-RELATED
DISEASES

Inventor(s): HIROYUKI SAITO et al.
DOCKET NO.: 053466-0325

107/589501

Fig. 4

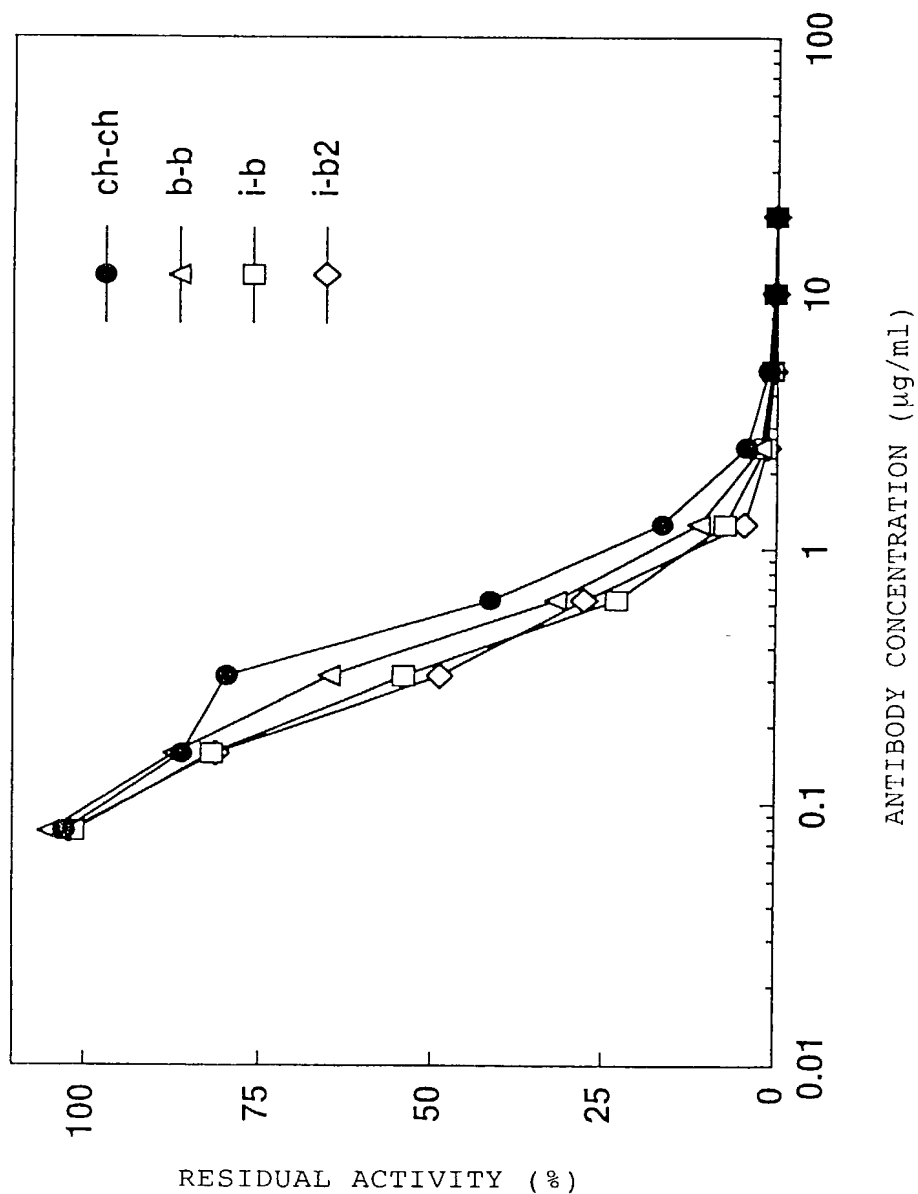


Fig. 5

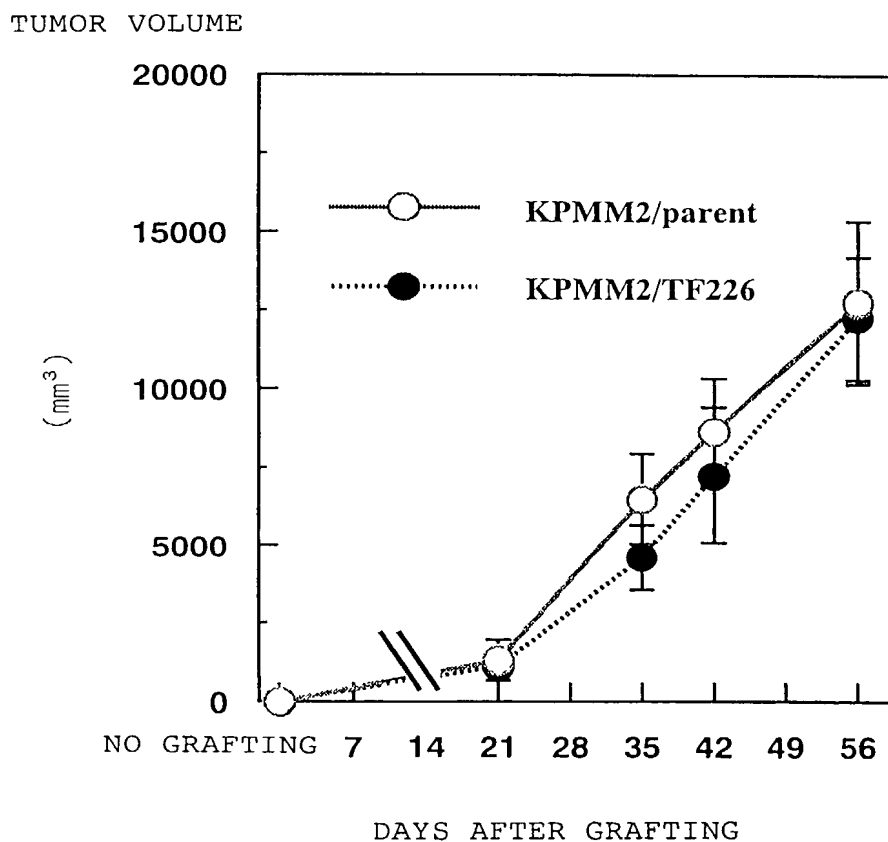
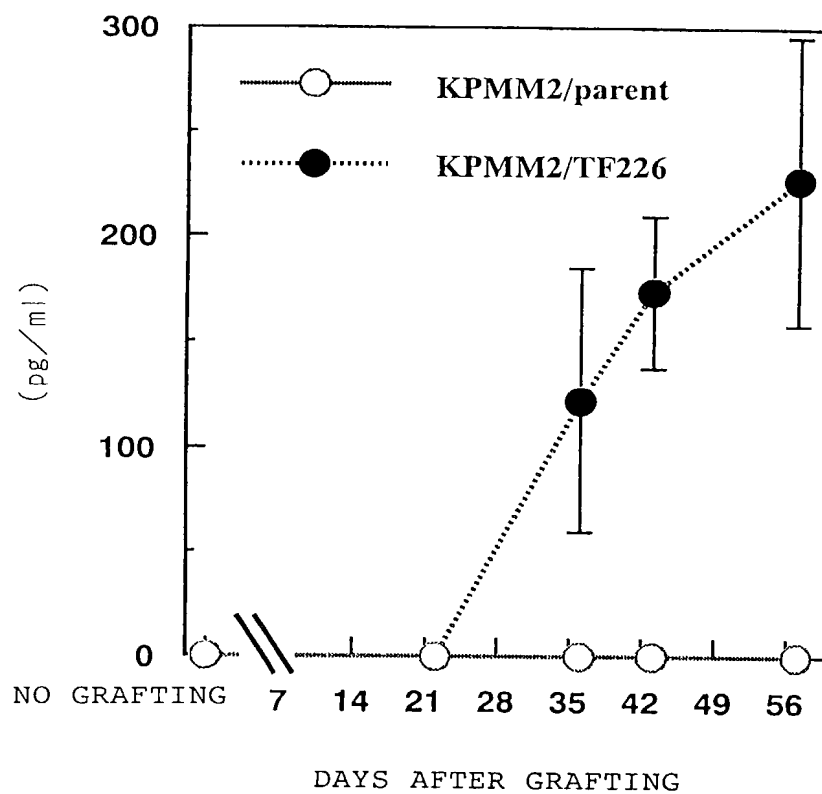


Fig. 6

hTF CONCENTRATION



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Fig.7

NUMBER OF PLATELETS

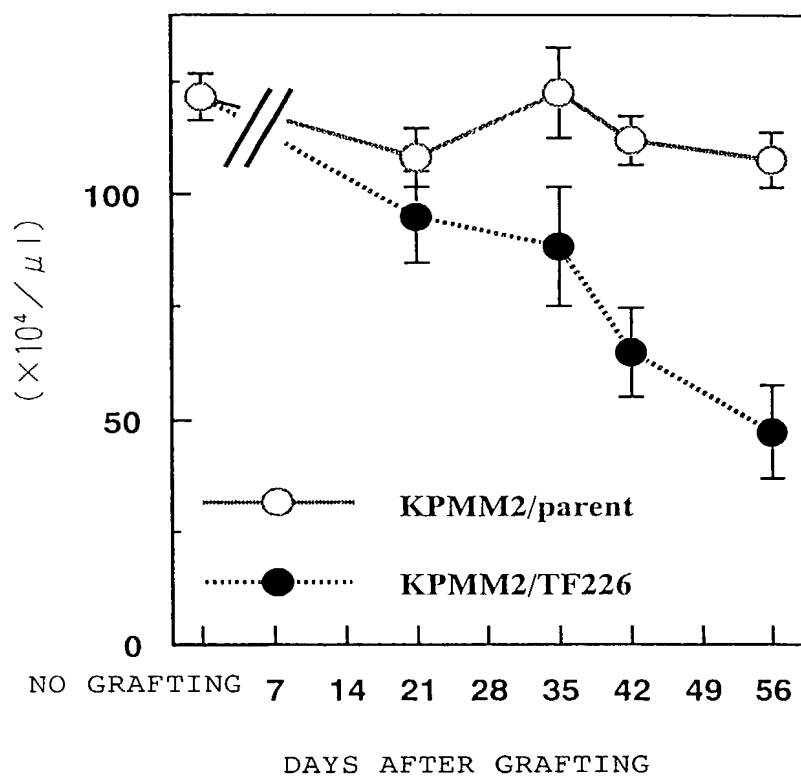


Fig. 8

FIBRINOGEN

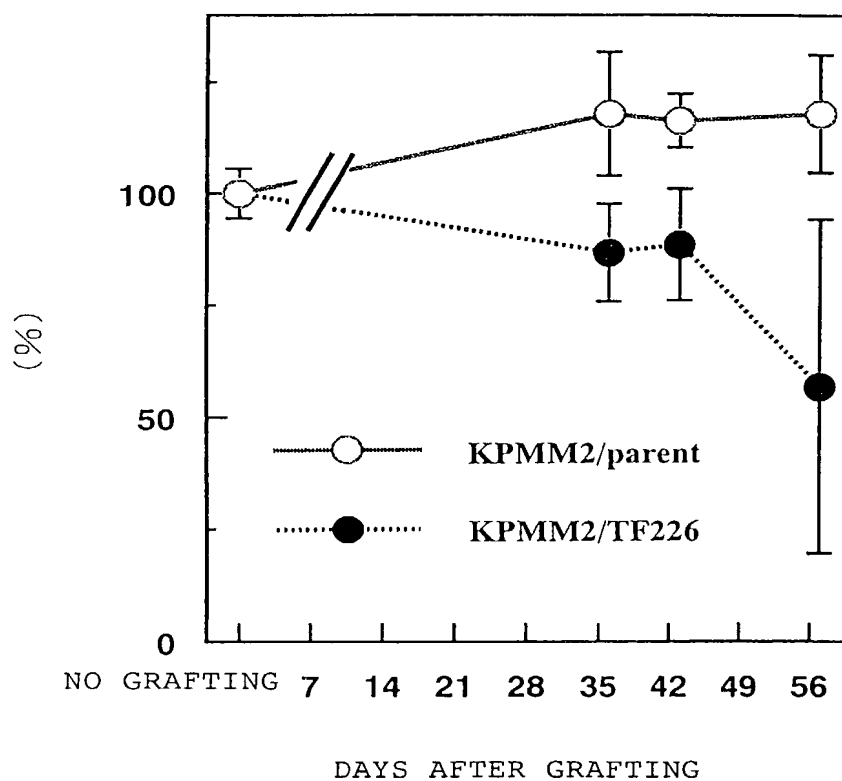


Fig.9

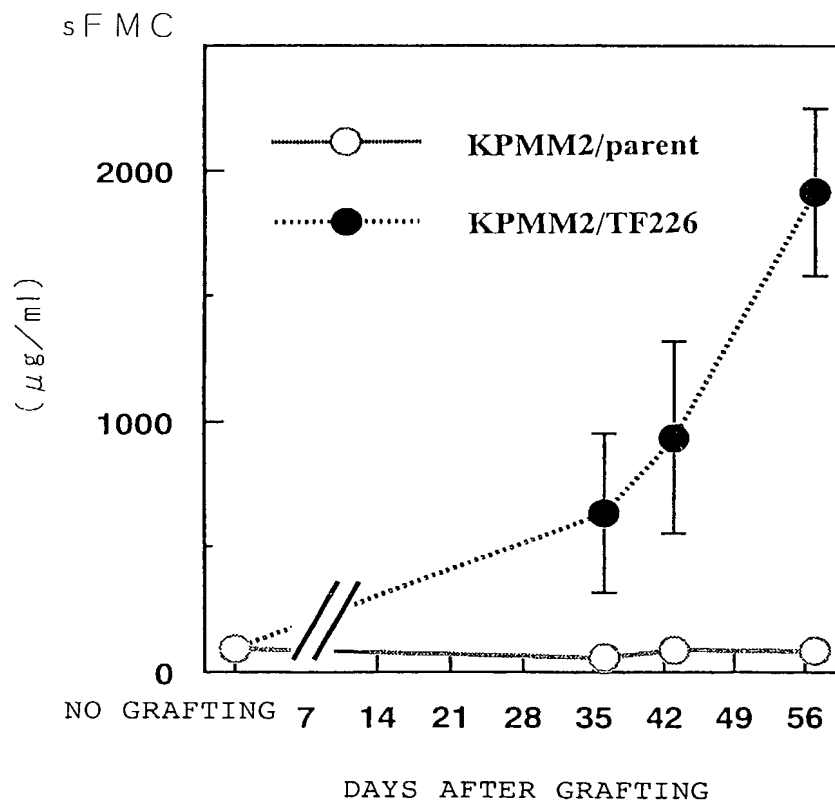
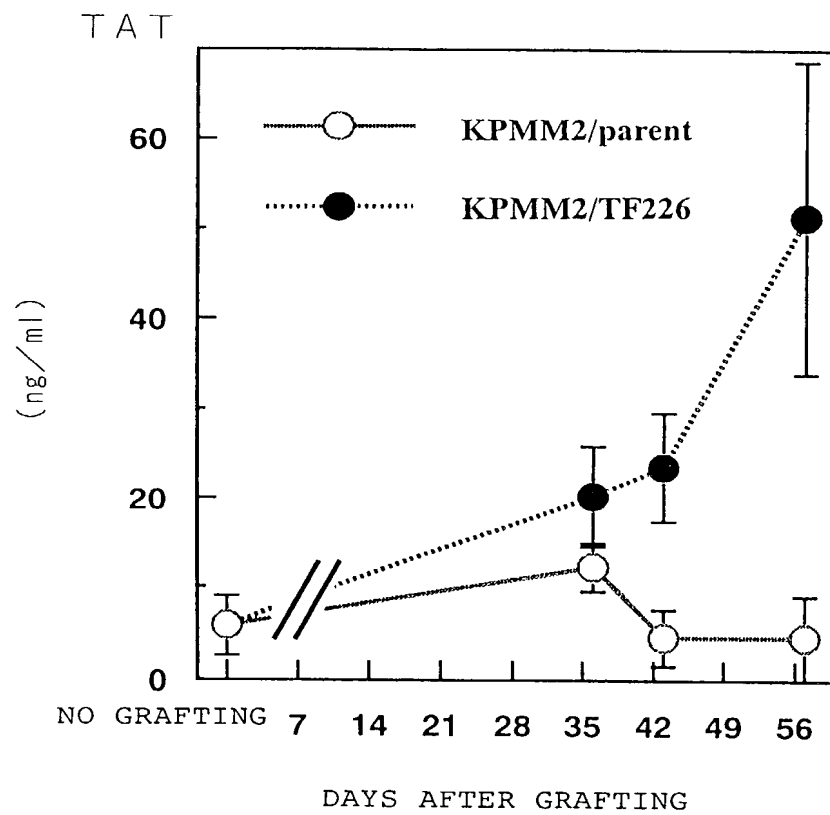


Fig.10



Title: PREVENTION AND TREATMENT
OF BLOOD COAGULATION-RELATED
DISEASES

Inventor(s): Hiroyuki SAITO et al.
DOCKET NO.: 053466-0325

Fig.11

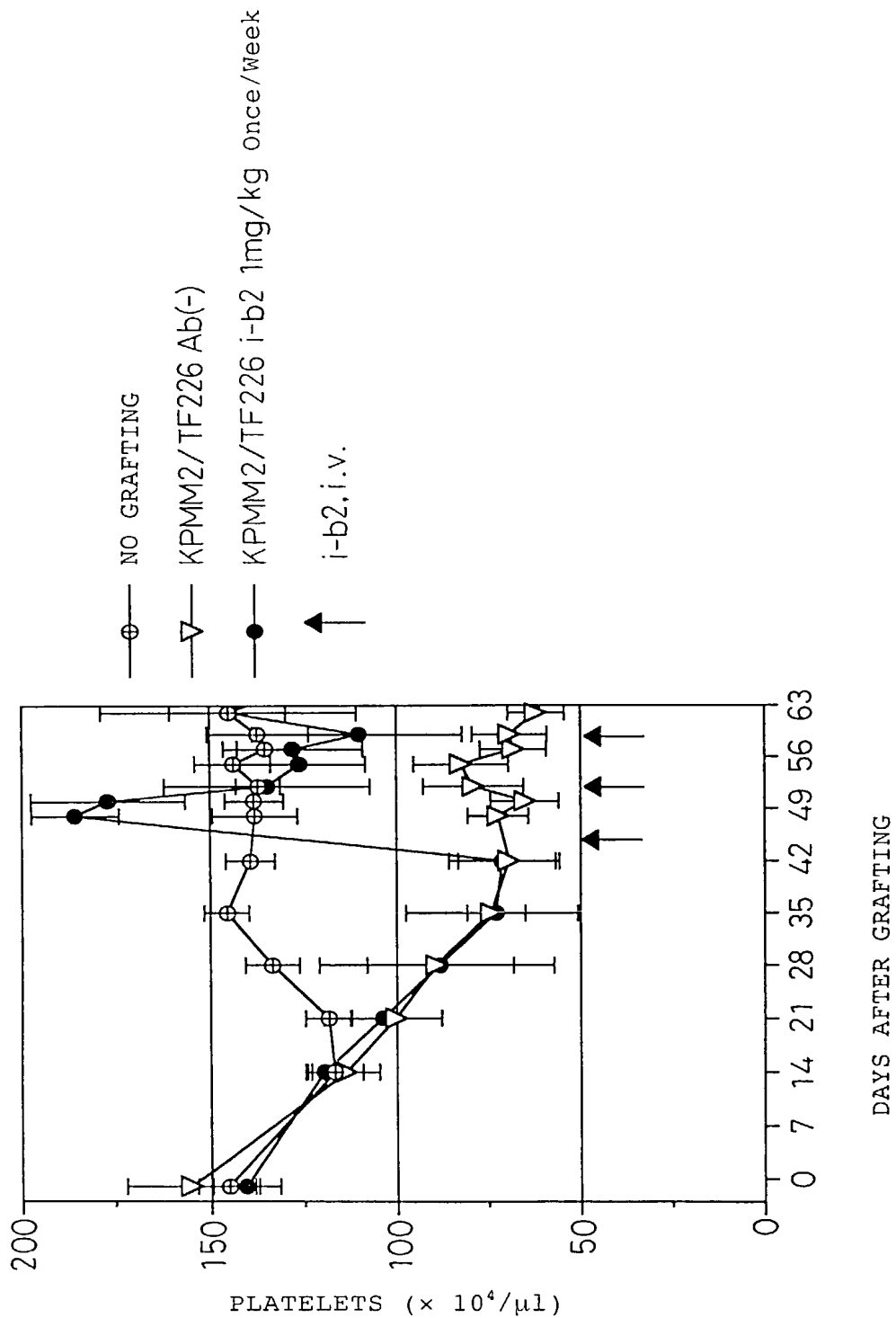


Fig.12

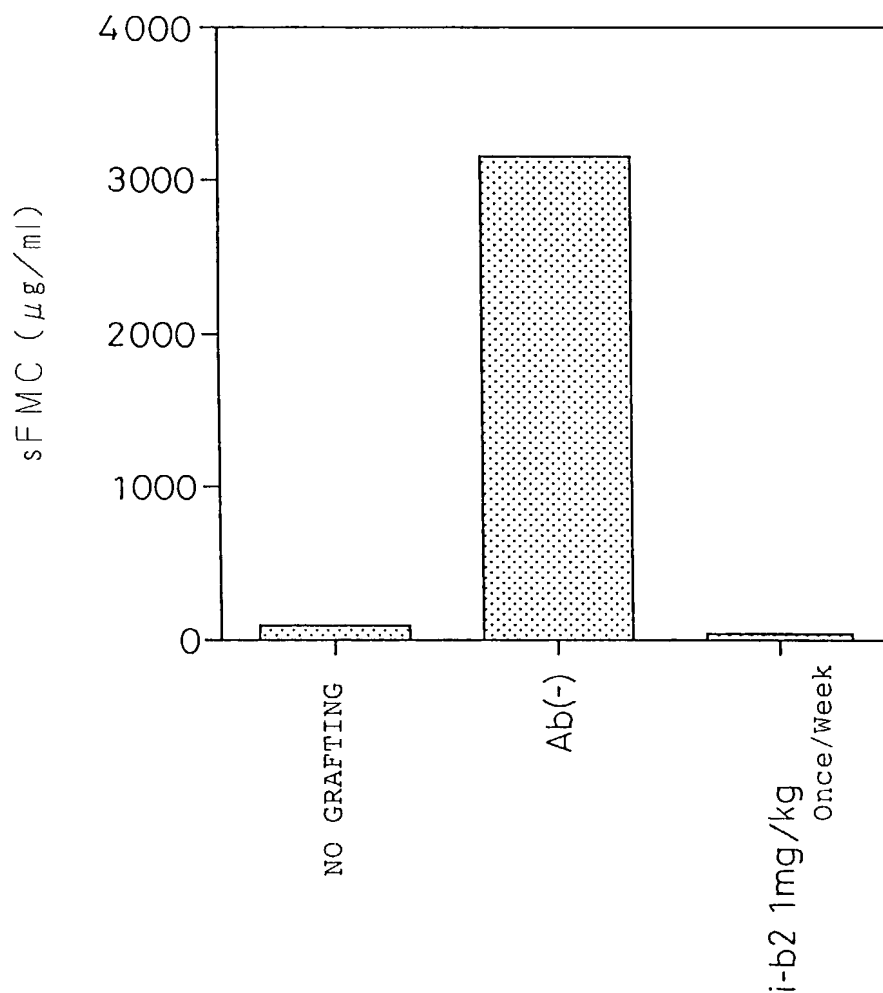


Fig.13

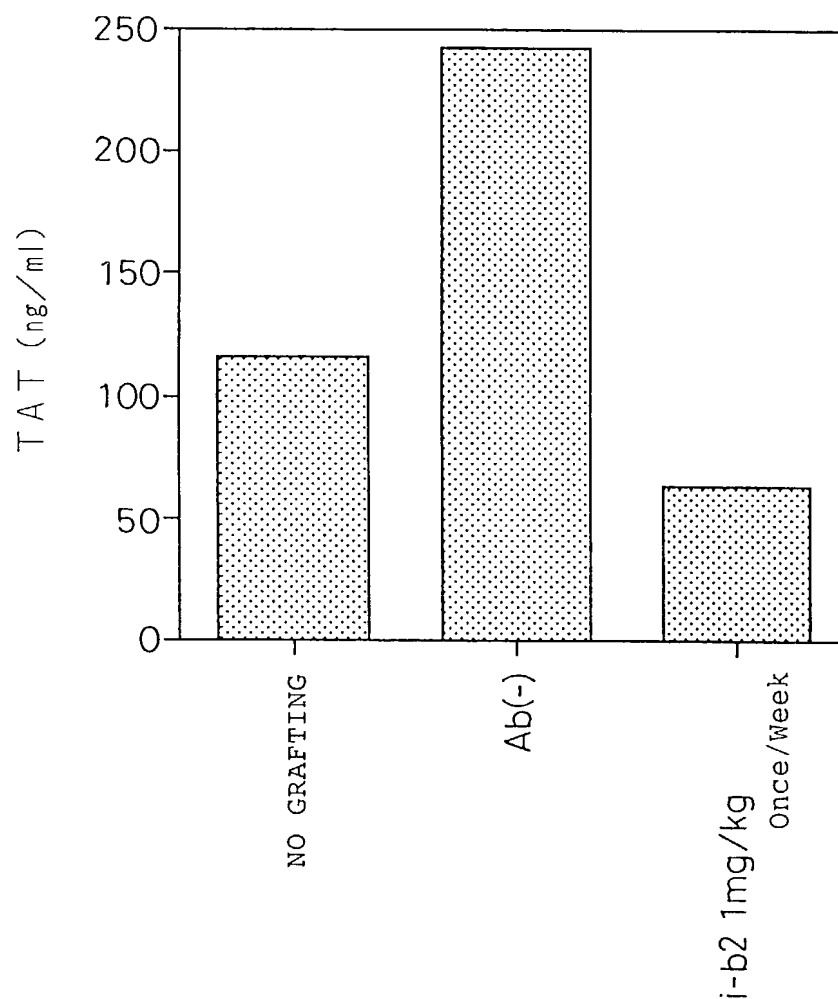
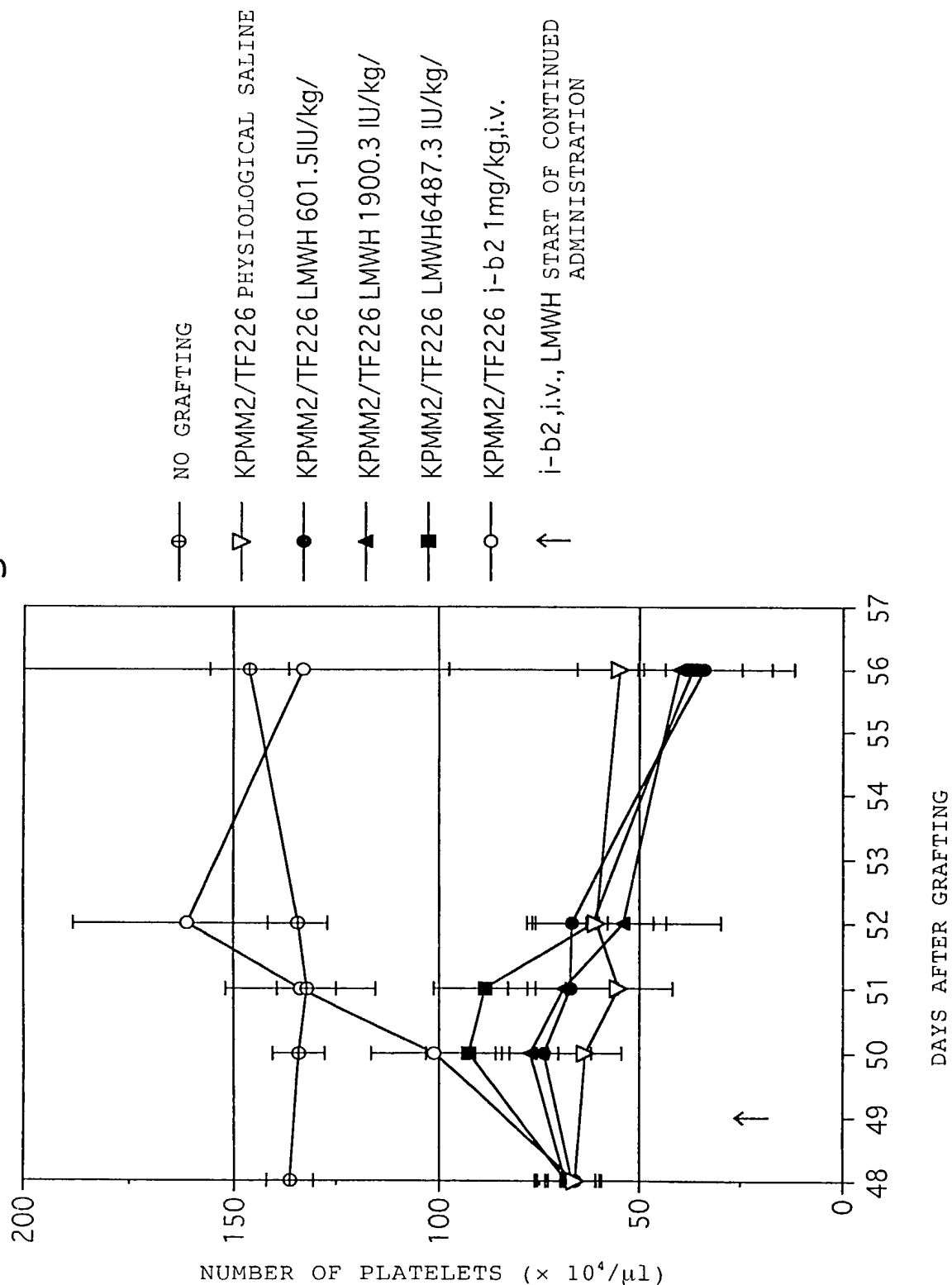


Fig.14



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I hereby claim foreign priority under Title 35, United States Code, Section 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

優先權主張

☒ Yes
はい

☐ No
いいえ

☒ Yes はい

☐ No いいえ

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(出願日)

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(現況: 特許許可済、係属中、放棄済)

(Status: Patented, Pending, Abandoned)
(現況: 特許許可済、係属中、放棄済)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Japanese Language Declaration (日本語宣言書)

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手続きを米特許商標局に対して遂行する弁理士または代理人
として、下記の者を指名いたします。(弁理士、または代理
人の氏名及び登録番号を明記のこと)

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18

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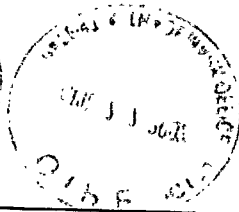
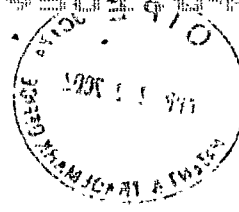
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(Supply similar information and signature for third and
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Japanese Language Declaration

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30

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同発明者の署名	Inventor's signature	Date
住所	Residence	
国籍	Citizenship	
郵便の宛先	Post Office Address	

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Pro	Lys	Phe	Gln	Gly	Lys	Ala	Ser	Ile	Thr	Ala	Asp	Thr	Ser	Ser	Asn	
			65					70					75			

aca gcc tac ctg cag ctc agc agc ctg aca tct gag gac act gcc gtc 336
 Thr Ala Tyr Leu Gln Leu Ser Ser Leu Thr Ser Glu Asp Thr Ala Val
 80 85 90

tat ttc tgt gct aga gac tcg ggc tat gct atg gac tac tgg ggt caa 384
 Tyr Phe Cys Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln
 95 100 105

gga acc tca gtc acc gtc tcc tca 408
 Gly Thr Ser Val Thr Val Ser Ser
 110 115

<210> 6
 <211> 381
 <212> DNA
 <213> Mouse
 <220>
 <221> sig-peptide
 <222> (1)...(60)
 <220>
 <221> mat-peptide
 <222> (61)...(381)
 <223> Nucleotide sequence coding for L chain V region of
 anti-TF mouse monoclonal antibody ATR-5
 <400> 6

atg agg gcc cct gct cag ttt ttt ggg atc ttg ttg ctc tgg ttt cca 48
 Met Arg Ala Pro Ala Gln Phe Phe Gly Ile Leu Leu Leu Trp Phe Pro
 -20 -15 -10 -5

ggg atc aga tgt gac atc aag atg acc cag tct cca tcc tct atg tat 96
 Gly Ile Arg Cys Asp Ile Lys Met Thr Gln Ser Pro Ser Ser Met Tyr
 1 5 10

gca tcg ctg gga gag aga gtc act atc act tgc aag gcg agt cag gac 144
 Ala Ser Leu Gly Glu Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp
 15 20 25

att aaa agc ttt tta agt tgg tac cag caa aaa cca tgg aaa tct cct 192
 Ile Lys Ser Phe Leu Ser Trp Tyr Gln Gln Lys Pro Trp Lys Ser Pro
 30 35 40

aag	acc	ctg	atc	tat	tat	gca	aca	agc	ttg	gca	gat	ggg	gtc	cca	tca	240
Lys	Thr	Leu	Ile	Tyr	Tyr	Ala	Thr	Ser	Leu	Ala	Asp	Gly	Val	Pro	Ser	
45					50					55					60	
aga	ttc	agt	ggc	agt	gga	tct	ggg	caa	gat	tat	tct	cta	acc	atc	aac	288
Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Gln	Asp	Tyr	Ser	Leu	Thr	Ile	Asn	
				65					70					75		
aac	ctg	gag	tct	gac	gat	aca	gca	act	tat	tat	tgt	cta	cag	cat	ggc	336
Asn	Leu	Glu	Ser	Asp	Asp	Thr	Ala	Thr	Tyr	Tyr	Cys	Leu	Gln	His	Gly	
			80					85					90			
gag	agc	ccg	tac	acg	ttc	gga	ggg	ggg	acc	aag	ctg	gaa	ata	aaa		381
Glu	Ser	Pro	Tyr	Thr	Phe	Gly	Gly	Gly	Thr	Lys	Leu	Glu	Ile	Lys		
		95					100					105				

<210> 7

<211> 35

<212> DNA

<213> Artificial Sequence

 $\langle 220 \rangle$

<223> Primer ch5HS

<400> 7

gtctgtcgac ccaccatgaa atgcagctgg gtcac 35

<210> 8

<211> 28

<212> DNA

<213> Artificial Sequence

 $\langle 220 \rangle$

<223> Primer ch5HA

<400> 8

tgttgctagc tgaggagacg gtgactga 28

$\langle 210 \rangle$ 9

$\langle 211 \rangle$ 35

<212> DNA

<213> Artificial Sequence

 $\langle 220 \rangle$

<223> Primer ch5LS

<400> 9

gtctagatct ccaccatgag ggcccctgct cagtt 35

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<210> 10
<211> 28
<212> DNA
<213> Artificial Sequence
<220>
<223> Primer ch5LA
<400> 10
tggttcgtacg ttttatttcc agcttggt                28
<210> 11
<211> 104
<212> DNA
<213> Artificial Sequence
<220>
<223> CDR grafting primer hR5Hv1S
<400> 11
ttctgtcgac ccaccatgaa atgcagctgg gtcattcttct tcttgatggc agtggttaca 60
gggggttaact cacaggtgca gctgttgagg tctggagctg tgct                104
<210> 12
<211> 108
<212> DNA
<213> Artificial Sequence
<220>
<223> CDR grafting primer hR5Hv28
<400> 12
acaggtgcag ctgttgagg ctggagctgt gctggcaagg cctgggactt ccgtgaagat 60
ctctcgcaag gcttccgat tcaacattaa agactactat atgcattg                108
<210> 13
<211> 108
<212> DNA
<213> Artificial Sequence
<220>
<223> CDR grafting primer hR5Hv4S
<400> 13
gaatggccat agtatgtatg acccgaaatt ccagggcagg gccaaactga ctgcagccac 60
atccgccagt attgcctact tggagttctc gagcctgaca aatgagga                108

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<210> 14
 <211> 110
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> CDR grafting primer hr5Hv3A
 <400> 14
 tcatacatatc tatggccatt cgcaggatca ttcccaccaa tccattctag accctgtcca 60
 ggcctctgtt ttacccaatg catatagtag tctttaatgt tgaatccgga 110
 <210> 15
 <211> 110
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> CDR grafting primer hr5Hv5A
 <400> 15
 agaagctagc tgaggagacg gtgaccaggg tgccttggcc ccagtagtcc atggcatagc 60
 ccgagtctct tgcacagtaa tagaccgcag aatcctcatt tgtcaggctc 110
 <210> 16
 <211> 19
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> Primer hr5HvPrS
 <400> 16
 ttctgtcgac ccaccatga 19
 <210> 17
 <211> 19
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> Primer hr5HvPrA
 <400> 17
 agaagctagc tgaggagac 19
 <210> 18

<211> 415

<212> DNA

<213> Artificial Sequence

<220>

<221> sig-peptide

<222> (1)...(57)

<220>

<221> mat-peptide

<222> (58)...(415)

<223> Nucleotide sequence coding for version "a" of
humanized H chain V region

<400> 18

atg aaa tgc agc tgg gtc atc ttc ttc ctg atg gca gtg gtt aca ggg	48
Met Lys Cys Ser Trp Val Ile Phe Phe Leu Met Ala Val Val Thr Gly	
-15 -10 -5	
gtt aac tca cag gtg cag ctg ttg gag tct gga gct gtg ctg gca agg	96
Val Asn Ser Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg	
1 5 10	
cct ggg act tcc gtg aag atc tcc tgc aag gct tcc gga ttc aac att	144
Pro Gly Thr Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile	
15 20 25	
aaa gac tac tat atg cat tgg gta aaa cag agg cct gga cag ggt cta	192
Lys Asp Tyr Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu	
30 35 40 45	
gaa tgg att ggt ggg aat gat cct gcg aat ggc cat agt atg tat gac	240
Glu Trp Ile Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp	
50 55 60	
ccg aaa ttc cag ggc agg gcc aaa ctg act gca gcc aca tcc gcc agt	288
Pro Lys Phe Gln Gly Arg Ala Lys Leu Thr Ala Ala Thr Ser Ala Ser	
65 70 75	
att gcc tac ttg gag ttc tcg agc ctg aca aat gag gat tct gcg gtc	336
Ile Ala Tyr Leu Glu Phe Ser Ser Leu Thr Asn Glu Asp Ser Ala Val	
80 85 90	
tat tac tgt gca aga gac tcg ggc tat gcc atg gac tac tgg ggc caa	384
Tyr Tyr Cys Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln	
95 100 105	

415

Gly Thr Leu Val Thr Val Ser Ser Ala Ser

110 115

<210> 19

<211> 119

<212> PRT

<213> Artificial Sequence

 $\langle 220 \rangle$

<223> Amino acid sequence of version "a" of humanized H chain V region

<400> 19

Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg Pro Gly Thr

1 5 10 15

Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile Lys Asp Tyr

20 25 30

Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile

35 40 45

Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp Pro Lys Phe

50 55 60

Gln Gly Arg Ala Lys Leu Thr Ala Ala Thr Ser Ala Ser Ile Ala Tyr

65 70 75 80

Leu Glu Phe Ser Ser Leu Thr Asn Glu Asp Ser Ala Val Tyr Tyr Cys

85 90 95

Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu

100 105 110

Val Thr Val Ser Ser Ala Ser

115

 $\langle 210 \rangle$ 20 $\langle 211 \rangle$ 100

<212> DNA

<213> Artificial Sequence

 $\langle 220 \rangle$

<223> FR Shuffling primer F3RFFS

<400> 20

ttcttggcca tagtatgtat gacccgaaat tccagggccg agtcacaatc actgcagaca 60

catccacgaa cacagcctac atggagctct cgagtctgag 100


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<210> 21
<211> 75
<212> DNA
<213> Artificial Sequence
<220>
<223> FR Shuffling primer F3RFBS
<400> 21
ggagctctcg agtctgagat ctgaggacac agccatttat tactgtgcaa gagactcggg 60
ctatgccatg gttct 75
<210> 22
<211> 100
<212> DNA
<213> Artificial Sequence
<220>
<223> FR Shuffling primer F3RFFA
<400> 22
ctcagactcg agagctccat gtaggctgtg ttogtggatg tgtctgcagt gattgtgact 60
cggccctgga atttcggggtc atacatacta tggccaagaa 100
<210> 23
<211> 75
<212> DNA
<213> Artificial Sequence
<220>
<223> FR Shuffling primer F3RFBA
<400> 23
agaacccatgg catagcccga gtctcttgca cagtaataaaa tggctgtgtc ctcagatctc 60
agactcgaga gctcc 75
<210> 24
<211> 100
<212> DNA
<213> Artificial Sequence
<220>
<223> FR Shuffling primer F3NMFS
<400> 24
ttcttggcca tagtatgtat gacccgaaat tccagggccg agtcacaatg ctggtagaca 60

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catccaagaa ccagttctcc ctgaggctct cgagtgtgac 100
 <210> 25
 <211> 75
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> FR Shuffling primer F3NMBS
 <400> 25
 gaggtctctg agtgtgacag ccgcggacac agccgtatat tactgtgcaa gagactcggg 60
 ctatgccatg gttct 75
 <210> 26
 <211> 100
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> FR Shuffling primer F3NMFA
 <400> 26
 gtcacactcg agagcctcag ggagaactgg ttcttggatg tgtctaccag cattgtgact 60
 cggccctgga atttcgggtc atacatacta tggccaagaa 100
 <210> 27
 <211> 75
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> FR Shuffling primer F3NMBA
 <400> 27
 agaaccatgg catagcccga gtctcttgca cagtaatata cggctgtgtc cgcggctgtc 60
 aactcggaga gcctc 75
 <210> 28
 <211> 414
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221> sig-peptide
 <222> (1)...(57)

<220>

<221> mat-peptide

<222> (58)...(414)

<223> Nucleotide sequence coding for version "b" of
humanized H chain V region

<400> 28

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atg aaa tgc agc tgg gtc atc ttc ttc ctg atg gca gtg gtt aca ggg      48
Met Lys Cys Ser Trp Val Ile Phe Phe Leu Met Ala Val Val Thr Gly
                    -15                    -10                    -5

gtt aac tca cag gtg cag ctg ttg gag tct gga gct gtg ctg gca agg      96
Val Asn Ser Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg
                    1                    5                    10

cct ggg act tcc gtg aag atc tcc tgc aag gct tcc gga ttc aac att     144
Pro Gly Thr Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile
                    15                    20                    25

aaa gac tac tat atg cat tgg gta aaa cag agg cct gga cag ggt cta     192
Lys Asp Tyr Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu
                    30                    35                    40                    45

gaa tgg att ggt ggg aat gat cct gcg aat ggc cat agt atg tat gac     240
Glu Trp Ile Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp
                    50                    55                    60

ccg aaa ttc cag ggc cga gtc aca atc act gca gac aca tcc acg aac     288
Pro Lys Phe Gln Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Asn
                    65                    70                    75

aca gcc tac atg gag ctc tcg agt ctg aga tct gag gac aca gcc att     336
Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Ile
                    80                    85                    90

tat tac tgt gca aga gac tcg ggc tat gcc atg gac tac tgg ggc caa     384
Tyr Tyr Cys Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln
                    95                    100                    105

ggc acc ctg gtc acc gtc tcc tca gct agc                                414
Gly Thr Leu Val Thr Val Ser Ser Ala Ser
110                                115

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<210> 29

<211> 119

<212> PRT

<213> Artificial Sequence

[illegible]

$\langle 211 \rangle$ 414

<213> Artificial Sequence

<221> sig-peptide

<222> (1) . . . (57)

 $\langle 220 \rangle$

<221> mat-peptide

 $\langle 222 \rangle \quad (58) \dots (414)$

<223> Nucleotide sequence coding for version "c" of humanized H chain V region

 $\langle 400 \rangle$ 30

atg aaa tgc agc tgg gtc atc ttc ttc ctg atg gca gtg gtt aca ggg 48
Met Lys Cys Ser Trp Val Ile Phe Phe Leu Met Ala Val Val Thr Gly
-15 -10 -5

Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
 35 40 45
 Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp Pro Lys Phe
 50 55 60
 Gln Gly Arg Val Thr Met Leu Val Asp Thr Ser Lys Asn Gln Phe Ser
 65 70 75 80
 Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr Cys
 85 90 95
 Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu
 100 105 110
 Val Thr Val Ser Ser Ala Ser
 115

<210> 32

<211> 100

<212> DNA

<213> Artificial Sequence

<220>

<223> FR Shuffling primer F3EPS

<400> 32

ttcttgcca tagtatgtat gacccgaaat tccagggcag agtcacgatt actgcggacg 60
 aatccacgag cacagcctac atggagctct cgagtctgag 100

<210> 33

<211> 75

<212> DNA

<213> Artificial Sequence

<220>

<223> FR Shuffling primer F3EPA

<400> 33

agaaccatgg catagcccga gtctctcgca cagaaatata cggccgagtc ctcagatctc 60
 agactcgaga gctcc 75

<210> 34

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer F3PrS

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<400> 34
ttcttggcca tagtatgtat 20
<210> 35
<211> 18
<212> DNA
<213> Artificial Sequence
<220>
<223> Primer F3PrA
<400> 35
agaaccatgg catagccc 18
<210> 36
<211> 100
<212> DNA
<213> Artificial Sequence
<220>
<223> FR Shuffling primer F3VHS
<400> 36
ttcttggcca tagtatgtat gacccgaaat tccagggcag agtctcgatt accgcggacg 60
agtcaacgaa gatagcctac atggagctca acagtctgag 100
<210> 37
<211> 75
<212> DNA
<213> Artificial Sequence
<220>
<223> FR Shuffling primer F3VHA
<400> 37
agaaccatgg catagcccga gtctctcgca cagaaataaa cggccgtgtc ctcagatctc 60
agactgttga gctcc 75
<210> 38
<211> 414
<212> DNA
<213> Artificial Sequence
<220>
<221> sig-peptide
<222> (1)...(57)

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<223> Amino acid sequence of version "d" of humanized H chain

[illegible]

$\langle 211 \rangle$ 414

<212> DNA

<213> Artificial Sequence

 $\langle 220 \rangle$

<221> sig-peptide

<222> (1) . . . (57)

 $\langle 220 \rangle$

<221> mat-peptide

 $\langle 222 \rangle \quad (58) \dots (414)$

<223> Nucleotide sequence coding for version "e" of humanized H chain V region

<400> 40

atg aaa tgc agc tgg gtc atc ttc ttc ctg atg gca gtg gtt aca ggg 48
Met Lys Cys Ser Trp Val Ile Phe Phe Leu Met Ala Val Val Thr Gly
-15 -10 -5

Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg Pro Gly Thr
1 5 10 15
Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile Lys Asp Tyr
20 25 30

Tyr	Met	His	Trp	Val	Lys	Gln	Arg	Pro	Gly	Gln	Gly	Leu	Glu	Trp	Ile		
35				40						45							
Gly	Gly	Asn	Asp	Pro	Ala	Asn	Gly	His	Ser	Met	Tyr	Asp	Pro	Lys	Phe		
50				55						60							
Gln	Gly	Arg	Val	Ser	Ile	Thr	Ala	Asp	Glu	Ser	Thr	Lys	Ile	Ala	Tyr		
65				70						75						80	
Met	Glu	Leu	Asn	Ser	Leu	Arg	Ser	Glu	Asp	Thr	Ala	Val	Tyr	Phe	Cys		
				85						90						95	
Ala	Arg	Asp	Ser	Gly	Tyr	Ala	Met	Asp	Tyr	Trp	Gly	Gln	Gly	Thr	Leu		
				100						105						110	
Val	Thr	Val	Ser	Ser	Ala	Ser											
115																	

$\langle 210 \rangle$ 42

 $\langle 211 \rangle$ 100

<212> DNA

<213> Artificial Sequence

 $\langle 220 \rangle$

<223> FR Shuffling primer F3SSS

<400> 42

ttottggcca tagtatgtat gacccgaaat tccagggcag agtcacgatt accgcggaca 60
catccaacgag cacagcctac atggagctca ggagcctgag 100

<210> 43

<211> 75

<212> DNA

<213> Artificial Sequence

 $\langle 220 \rangle$

<223> FR Shuffling primer F3SSA

<400> 43

agaacccatgg catagcccga gtctctcgca cagtaataca cggccgtgtc gtcagatctc 60
aggctcctga qctcc 75

<210> 44

 $\langle 211 \rangle$ 100

<212> DNA

<213> Artificial Sequence

 $\langle 220 \rangle$

<223> FR Shuffling primer F3CDS

<400> 44
 ttcttgccca tagtatgtat gacccgaaat tccagggcaa agccactctg actgcagacg 60
 aatcctccag cacagcctac atgcaactct cgagcctacg 100
 <210> 45
 <211> 75
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> FR Shuffling primer F3CDA
 <400> 45
 agaaccatgg catagcccga gtctcttgca caagaataga ccgcagagtc ctcagatcgt 60
 aggctcgaga gttgc 75
 <210> 46
 <211> 414
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221> sig-peptide
 <222> (1)...(57)
 <220>
 <221> mat-peptide
 <222> (58)...(414)
 <223> Nucleotide sequence coding for version "f" of
 humanized H chain V region
 <400> 46
 atg aaa tgc agc tgg gtc atc ttc ttc ctg atg gca gtg gtt aca ggg 48
 Met Lys Cys Ser Trp Val Ile Phe Phe Leu Met Ala Val Val Thr Gly
 -15 -10 -5
 gtt aac tca cag gtg cag ctg ttg gag tct gga gct gtg ctg gca agg 96
 Val Asn Ser Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg
 1 5 10
 cct ggg act tcc gtg aag atc tcc tgc aag gct tcc gga ttc aac att 144
 Pro Gly Thr Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile
 15 20 25

aaa	gac	tac	tat	atg	cat	tgg	gta	aaa	cag	agg	cct	gga	cag	ggt	cta	192
Lys	Asp	Tyr	Tyr	Met	His	Trp	Val	Lys	Gln	Arg	Pro	Gly	Gln	Gly	Leu	
30						35				40					45	
gaa	tgg	att	ggg	ggg	aat	gat	cct	gcg	aat	ggc	cat	agt	atg	tat	gac	240
Glu	Trp	Ile	Gly	Gly	Asn	Asp	Pro	Ala	Asn	Gly	His	Ser	Met	Tyr	Asp	
				50					55					60		
ccg	aaa	ttc	cag	ggc	aga	gtc	acg	att	acc	gcg	gac	aca	tcc	acg	agc	288
Pro	Lys	Phe	Gln	Gly	Arg	Val	Thr	Ile	Thr	Ala	Asp	Thr	Ser	Thr	Ser	
			65					70					75			
aca	gcc	tac	atg	gag	ctc	agg	agc	ctg	aga	tct	gac	gac	acg	gcc	gtg	336
Thr	Ala	Tyr	Met	Glu	Leu	Arg	Ser	Leu	Arg	Ser	Asp	Asp	Thr	Ala	Val	
		80						85				90				
tat	tac	tgt	gcg	aga	gac	tcg	ggc	tat	gcc	atg	gac	tac	tgg	ggc	caa	384
Tyr	Tyr	Cys	Ala	Arg	Asp	Ser	Gly	Tyr	Ala	Met	Asp	Tyr	Trp	Gly	Gln	
	95					100					105					
ggc	acc	ctg	gtc	acc	gtc	tcg	tca	gct	agc							414
Gly	Thr	Leu	Val	Thr	Val	Ser	Ser	Ala	Ser							

<210> 47

<211> 119

<212> PRT

<213> Artificial Sequence

 $\langle 220 \rangle$

<223> Amino acid sequence of version "f" of humanized H chain V region

<400> 47

Gln	Val	Gln	Leu	Leu	Glu	Ser	Gly	Ala	Val	Leu	Ala	Arg	Pro	Gly	Thr
1				5					10					15	
Ser	Val	Lys	Ile	Ser	Cys	Lys	Ala	Ser	Gly	Phe	Asn	Ile	Lys	Asp	Tyr
			20					25					30		
Tyr	Met	His	Trp	Val	Lys	Gln	Arg	Pro	Gly	Gln	Gly	Leu	Glu	Trp	Ile
			35				40						45		
Gly	Gly	Asn	Asp	Pro	Ala	Asn	Gly	His	Ser	Met	Tyr	Asp	Pro	Lys	Phe
		50					55				60				
Gln	Gly	Arg	Val	Thr	Ile	Thr	Ala	Asp	Thr	Ser	Thr	Ser	Thr	Ala	Tyr
65					70					75					80

```
<210> 48
<211> 414
<212> DNA
<213> Artificial Sequence
<220>
<221> sig-peptide
<222> (1)...(57)
<220>
<221> mat-peptide
<222> (58)...(414)
<223> Nucleotide sequence
humanized H chain V regio
<400> 48
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22/57

ccg aaa ttc cag ggc aaa gcc act ctg act gca gac gaa tcc tcc agc 288
 Pro Lys Phe Gln Gly Lys Ala Thr Leu Thr Ala Asp Glu Ser Ser Ser
 65 70 75
 aca gcc tac atg caa ctc tcg agc cta cga tct gag gac tct gcg gtc 336
 Thr Ala Tyr Met Gln Leu Ser Ser Leu Arg Ser Glu Asp Ser Ala Val
 80 85 90
 tat tct tgt gca aga gac tcg ggc tat gcc atg gac tac tgg ggc caa 384
 Tyr Ser Cys Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln
 95 100 105
 ggc acc ctg gtc acc gtc tcc tca gct agc 414
 Gly Thr Leu Val Thr Val Ser Ser Ala Ser
 110 115

<210> 49

<211> 119

<212> PRT

<213> Artificial Sequence

<220>

<223> Amino acid sequence of version "g" of humanized H
 chain V region

<400> 49

Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg Pro Gly Thr
 1 5 10 15
 Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile Lys Asp Tyr
 20 25 30
 Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
 35 40 45
 Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp Pro Lys Phe
 50 55 60
 Gln Gly Lys Ala Thr Leu Thr Ala Asp Glu Ser Ser Ser Thr Ala Tyr
 65 70 75 80
 Met Gln Leu Ser Ser Leu Arg Ser Glu Asp Ser Ala Val Tyr Ser Cys
 85 90 95
 Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu
 100 105 110
 Val Thr Val Ser Ser Ala Ser
 115

<210> 50

10

cct	ggg	act	tcc	gtg	aag	atc	tcc	tgc	aag	gct	tcc	gga	ttc	aac	att	144	
Pro	Gly	Thr	Ser	Val	Lys	Ile	Ser	Cys	Lys	Ala	Ser	Gly	Phe	Asn	Ile		
15						20						25					
aaa	gac	tac	tat	atg	cat	tgg	gta	aaa	cag	agg	cct	gga	cag	ggt	cta	192	
Lys	Asp	Tyr	Tyr	Met	His	Trp	Val	Lys	Gln	Arg	Pro	Gly	Gln	Gly	Leu		
30						35						40			45		
gaa	tgg	att	ggt	ggg	aat	gat	cct	gcg	aat	ggc	cat	agt	atg	tat	gac	240	
Glu	Trp	Ile	Gly	Gly	Asn	Asp	Pro	Ala	Asn	Gly	His	Ser	Met	Tyr	Asp		
			50						55						60		
ccg	aaa	ttc	cag	ggc	cgc	gtc	acc	atg	tca	gcc	gac	aag	tcc	tcc	agc	288	
Pro	Lys	Phe	Gln	Gly	Arg	Val	Thr	Met	Ser	Ala	Asp	Lys	Ser	Ser	Ser		
			65						70						75		
gcc	gcc	tat	tta	cag	tgg	acc	agc	ctt	aag	gcc	tcg	gac	acc	gcc	ata	336	
Ala	Ala	Tyr	Leu	Gln	Trp	Thr	Ser	Leu	Lys	Ala	Ser	Asp	Thr	Ala	Ile		
80						85						90					
tat	ttc	tgc	gcg	aga	gac	tcg	ggc	tat	gcc	atg	gac	tac	tgg	ggc	caa	384	
Tyr	Phe	Cys	Ala	Arg	Asp	Ser	Gly	Tyr	Ala	Met	Asp	Tyr	Trp	Gly	Gln		
95						100						105					
ggc	acc	ctg	gtc	acc	gtc	tcc	tca	gct	agc							414	
Gly	Thr	Leu	Val	Thr	Val	Ser	Ser	Ala	Ser								
110						115											

<210> 53

<211> 119

<212> PRT

<213> Artificial Sequence

 $\langle 220 \rangle$

<223> Amino acid sequence of version "h" of humanized H chain V region

<400> 53

Gln	Val	Gln	Leu	Leu	Glu	Ser	Gly	Ala	Val	Leu	Ala	Arg	Pro	Gly	Thr
1				5					10					15	
Ser	Val	Lys	Ile	Ser	Cys	Lys	Ala	Ser	Gly	Phe	Asn	Ile	Lys	Asp	Tyr
			20				25						30		
Tyr	Met	His	Trp	Val	Lys	Gln	Arg	Pro	Gly	Gln	Gly	Leu	Glu	Trp	Ile
		35					40					45			
Gly	Gly	Asn	Asp	Pro	Ala	Asn	Gly	His	Ser	Met	Tyr	Asp	Pro	Lys	Phe
	50					55					60				

Gln Gly Arg Val Thr Met Ser Ala Asp Lys Ser Ser Ser Ala Ala Tyr
 65 70 75 80
 Leu Gln Trp Thr Ser Leu Lys Ala Ser Asp Thr Ala Ile Tyr Phe Cys
 85 90 95
 Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu
 100 105 110
 Val Thr Val Ser Ser Ala Ser
 115

<210> 54

<211> 100

<212> DNA

<213> Artificial Sequence

<220>

<223> FR Shuffling primer F3MMS

<400> 54

ttcttgcca tagtatgtat gacccgaaat tccagggcag agtcacgatt accgcggaca 60
 catcgacgag cacagtcttc atggaactga gcagcctgag 100

<210> 55

<211> 75

<212> DNA

<213> Artificial Sequence

<220>

<223> FR Shuffling primer F3MMA

<400> 55

agaaccatgg catagcccga gtctctcgca cagtaataca cggccgtgtc ttcagatctc 60
 aggctgctca gttcc 75

<210> 56

<211> 100

<212> DNA

<213> Artificial Sequence

<220>

<223> FR Shuffling primer F3BMS

<400> 56

ttcttgcca tagtatgtat gacccgaaat tccagggcag agtcaccttt accgcggaca 60
 catccgcgaa cacagcctac atggagtga ggagcctcag 100

<210> 57

<211> 75
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> FR Shuffling primer F3BMA
 <400> 57
 agaaccatgg catagcccgga gtctctcgca caataataaa cagccgtgtc tgcagatctg 60
 aggctcctca actcc 75

<210> 58
 <211> 414
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221> sig-peptide
 <222> (1)...(57)
 <220>
 <221> mat-peptide
 <222> (58)...(414)
 <223> Nucleotide sequence coding for version "i" of
 humanized H chain V region
 <400> 58

atg	aaa	tgc	agc	tgg	gtc	atc	ttc	ttc	ctg	atg	gca	gtg	gtt	aca	ggg	48
Met	Lys	Cys	Ser	Trp	Val	Ile	Phe	Phe	Leu	Met	Ala	Val	Val	Thr	Gly	
				-15					-10					-5		
gtt	aac	tca	cag	gtg	cag	ctg	ttg	gag	tct	gga	gct	gtg	ctg	gca	agg	96
Val	Asn	Ser	Gln	Val	Gln	Leu	Leu	Glu	Ser	Gly	Ala	Val	Leu	Ala	Arg	
			1				5						10			
cct	ggg	act	tcc	gtg	aag	atc	tcc	tgc	aag	gct	tcc	gga	ttc	aac	att	144
Pro	Gly	Thr	Ser	Val	Lys	Ile	Ser	Cys	Lys	Ala	Ser	Gly	Phe	Asn	Ile	
			15				20					25				
aaa	gac	tac	tat	atg	cat	tgg	gta	aaa	cag	agg	cct	gga	cag	ggg	cta	192
Lys	Asp	Tyr	Tyr	Met	His	Trp	Val	Lys	Gln	Arg	Pro	Gly	Gln	Gly	Leu	
			30				35					40			45	
gaa	tgg	att	ggt	ggg	aat	gat	cct	gcg	aat	ggc	cat	agt	atg	tat	gac	240
Glu	Trp	Ile	Gly	Gly	Asn	Asp	Pro	Ala	Asn	Gly	His	Ser	Met	Tyr	Asp	
							50					55			60	

ccg aaa ttc cag ggc aga gtc acg att acc gcg gac aca tcg acg agc 288
 Pro Lys Phe Gln Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Ser
 65 70 75
 aca gtc ttc atg gaa ctg agc agc ctg aga tct gaa gac acg gcc gtg 336
 Thr Val Phe Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val
 80 85 90
 tat tac tgt gcg aga gac tcg ggc tat gcc atg gac tac tgg ggc caa 384
 Tyr Tyr Cys Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln
 95 100 105
 ggc acc ctg gtc acc gtc tcc tca gct agc 414
 Gly Thr Leu Val Thr Val Ser Ser Ala Ser
 110 115

<210> 59

<211> 119

<212> PRT

<213> Artificial Sequence

<220>

<223> Amino acid sequence of version "i" of humanized H
 chain V region

<400> 59

Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg Pro Gly Thr
 1 5 10 15
 Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile Lys Asp Tyr
 20 25 30
 Tyr Met His Trp Val Lys Gln Arg Pro Gly Gln Gly Leu Glu Trp Ile
 35 40 45
 Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp Pro Lys Phe
 50 55 60
 Gln Gly Arg Val Thr Ile Thr Ala Asp Thr Ser Thr Ser Thr Val Phe
 65 70 75 80
 Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
 85 90 95
 Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu
 100 105 110
 Val Thr Val Ser Ser Ala Ser
 115

<210> 60

$\langle 400 \rangle$ 60

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```
<210> 65
<211> 119
<212> PRT
<213> Artificial Sequence
<220>
<223> Amino acid sequence of version "b1" of humanized H
chain V region
```

Gln	Val	Gln	Leu	Leu	Glu	Ser	Gly	Ala	Val	Leu	Ala	Arg	Pro	Gly	Thr
1				5					10					15	
Ser	Val	Lys	Ile	Ser	Cys	Lys	Ala	Ser	Gly	Phe	Asn	Ile	Lys	Asp	Tyr
			20				25						30		
Tyr	Met	His	Trp	Val	Arg	Gln	Ala	Pro	Gly	Gln	Gly	Leu	Glu	Trp	Met
		35					40					45			
Gly	Gly	Asn	Asp	Pro	Ala	Asn	Gly	His	Ser	Met	Tyr	Asp	Pro	Lys	Phe
		50					55				60				
Gln	Gly	Arg	Val	Thr	Ile	Thr	Ala	Asp	Thr	Ser	Thr	Asn	Thr	Ala	Tyr
					70					75					80
Met	Glu	Leu	Ser	Ser	Leu	Arg	Ser	Glu	Asp	Thr	Ala	Ile	Tyr	Tyr	Cys
				85					90					95	
Ala	Arg	Asp	Ser	Gly	Tyr	Ala	Met	Asp	Tyr	Trp	Gly	Gln	Gly	Thr	Leu
			100					105					110		

115

$\langle 211 \rangle$ 414

<213> Artificial Sequence

 $\langle 220 \rangle$

<221> sig-peptide

<222> (1) . . . (57)

 $\langle 220 \rangle$

<221> mat-peptide

 $\langle 222 \rangle \quad (58) \dots (414)$

<223> Nucleotide sequence coding for version "d1" of humanized H chain V region

<400> 66

atg aaa tgc agc tgg gtc atc ttc ttc ctg atg gca gtg gtt aca ggg 48

Met Lys Cys Ser Trp Val Ile Phe Phe Leu Met Ala Val Val Thr Gly

-15

-10

-5

gtt aac tca caq gtg caq ctg ttg gag tct gga gct gtg ctg gca agg 96

Val Asn Ser Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg

1

5

10

cct ggg act tcc gtg aag atc tcc tgc aag gct tcc gga ttc aac att 144

Pro Gly Thr Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile

15

20

25

aaa gac tac tat atg cat tgg gtg cgc cag gct cca gga cag ggc ctg 192

Lys Asp Tyr Tyr Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu

30

35

40

45

qag tgg atg gga ggg aat gat cct gcg aat ggc cat agt atg tat gac 240

Glu Trp Met Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp

50

55

60

ccg aaa ttc cag ggc aga gtc acg att act gcg gac gaa tcc acg agc 288

Pro Lys Phe Gln Gly Arg Val Thr Ile Thr Ala Asp Glu Ser Thr Ser

65

70

75

aca gcc tac atg gag ctc tcg agt ctg aga tct gag gac tcg gcc gta 336

Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Ser Ala Val

80

85

90

tat ttc tgt gcg aga gac tcg ggc tat gcc atg gac tac tgg ggc caa 384
Tyr Phe Cys Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln

95 100 105

ggc acc ctg gtc acc gtc tcc tca gct agc 414

Gly Thr Leu Val Thr Val Ser Ser Ala Ser

110 115

<210> 67

<211> 119

<212> PRT

<213> Artificial Sequence

<220>

<223> Amino acid sequence of version "d1" of humanized H
chain V region

<400> 67

Gln Val Gln Leu Leu Glu Ser Gly Ala Val Leu Ala Arg Pro Gly Thr

1 5 10 15

Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Phe Asn Ile Lys Asp Tyr

20 25 30

Tyr Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met

35 40 45

Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp Pro Lys Phe

50 55 60

Gln Gly Arg Val Thr Ile Thr Ala Asp Glu Ser Thr Ser Thr Ala Tyr

65 70 75 80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Ser Ala Val Tyr Phe Cys

85 90 95

Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Leu

100 105 110

Val Thr Val Ser Ser Ala Ser

115

<210> 68

<211> 79

<212> DNA

<213> Artificial Sequence

<220>

<223> FR shuffling primer F2VHS

25

[illegible]

<210> 71

<211> 119

<212> PRT

<213> Artificial Sequence

 $\langle 220 \rangle$

<223> Amino acid sequence of version "b3" of humanized H chain V region

<400> 71

Gln	Val	Gln	Leu	Leu	Glu	Ser	Gly	Ala	Val	Leu	Ala	Arg	Pro	Gly	Thr
1				5					10					15	
Ser	Val	Lys	Ile	Ser	Cys	Lys	Ala	Ser	Gly	Phe	Asn	Ile	Lys	Asp	Tyr
			20					25					30		
Tyr	Met	His	Trp	Val	Arg	Gln	Ala	Pro	Gly	Gln	Gly	Leu	Glu	Trp	Ile
			35				40					45			
Gly	Gly	Asn	Asp	Pro	Ala	Asn	Gly	His	Ser	Met	Tyr	Asp	Pro	Lys	Phe
		50				55					60				
Gln	Gly	Arg	Val	Thr	Ile	Thr	Ala	Asp	Thr	Ser	Thr	Asn	Thr	Ala	Tyr
65					70						75				80

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<210> 72
<211> 414
<212> DNA
<213> Artificial Sequence
<220>
<221> sig-peptide
<222> (1)...(57)
<220>
<221> mat-peptide
<222> (58)...(414)
<223> Nucleotide sequence
humanized H chain V regio

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ccg	aaa	ttc	cag	ggc	aga	gtc	acg	att	act	gcg	gac	gaa	tcc	acg	agc	288
Pro	Lys	Phe	Gln	Gly	Arg	Val	Thr	Ile	Thr	Ala	Asp	Glu	Ser	Thr	Ser	
			65					70					75			
aca	gcc	tac	atg	gag	ctc	tcg	agt	ctg	aga	tct	gag	gac	tcg	gcc	gta	336
Thr	Ala	Tyr	Met	Glu	Leu	Ser	Ser	Leu	Arg	Ser	Glu	Asp	Ser	Ala	Val	
			80					85					90			
tat	ttc	tgt	gcg	aga	gac	tcg	ggc	tat	gcc	atg	gac	tac	tgg	ggc	caa	384
Tyr	Phe	Cys	Ala	Arg	Asp	Ser	Gly	Tyr	Ala	Met	Asp	Tyr	Trp	Gly	Gln	
		95					100					105				
ggc	acc	ctg	gtc	acc	gtc	tcc	tca	gct	agc							414
Gly	Thr	Leu	Val	Thr	Val	Ser	Ser	Ala	Ser							
110							115									

<210> 73

<211> 119

<212> PRT

<213> Artificial Sequence

 $\langle 220 \rangle$

<223> Amino acid sequence of version "d3" of humanized H chain V region

<400> 73

[illegible]

<210> 74

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<211> 98
<212> DNA
<213> Artificial Sequence
<220>
<223> FR shuffling vector h5Lv1S
<400> 74
gtctagatct ccaccatgag ggcctctgct cagttttttg ggatcttggt gctctgggtt 60
ccagggatcc gatgtgacat ccagatgacc cagtctcc 98
<210> 75
<211> 98
<212> DNA
<213> Artificial Sequence
<220>
<223> FR shuffling vector h5Lv4S
<400> 75
ttggcagatg gggctccatc aagggttcagt ggctccggat ctggtaccga tttcactctc 60
accatctcga gtctgcaacc tgaagatttt gcaactta 98
<210> 76
<211> 98
<212> DNA
<213> Artificial Sequence
<220>
<223> FR shuffling vector h5Lv2A
<400> 76
cttaagaagc ttttaatgtc ctgtgaggcc ttgcacgtga tggtgactct gtctcctaca 60
gatgcagaca gggaggatgg agactgggtc atctggat 98
<210> 77
<211> 98
<212> DNA
<213> Artificial Sequence
<220>
<223> FR shuffling vector h5Lv3A
<400> 77
gatgggaccc catctgccaa actagttgca taatagatca ggagcttagg ggctttccct 60
ggtttctgct gataccaact taagaagctt ttaatgtc 98

```


<400> 82

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
 1 5 10 15
 Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Ile Lys Ser Phe
 20 25 30
 Leu Ser Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
 35 40 45
 Tyr Tyr Ala Thr Ser Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
 50 55 60
 Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
 65 70 75 80
 Glu Asp Phe Ala Thr Tyr Tyr Cys Leu Gln His Gly Glu Ser Pro Tyr
 85 90 95
 Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
 100 105

<210> 83

<211> 77

<212> DNA

<213> Artificial Sequence

<220>

<223> FR shuffling primer F3SS

<400> 83

gtctggtacc gattacaetc tcaccatetc gagcctccag cctgaagatt ttgcaactta 60
 ctattgtctg cagaaca 77

<210> 84

<211> 77

<212> DNA

<213> Artificial Sequence

<220>

<223> FR shuffling primer F3SA

<400> 84

tggtctgcag acaatagtaa gttgcaaaat cttcaggctg gaggctcgag atggtgagag 60
 tgtaatcggt accagac 77

<210> 85

<211> 77

<212> DNA

<213> Artificial Sequence

25

att	aaa	agc	ttc	tta	agt	tgg	tat	cag	cag	aaa	cca	ggg	aaa	gcc	cct	192	
Ile	Lys	Ser	Phe	Leu	Ser	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Lys	Ala	Pro		
30						35						40					
aag	ctc	ctg	atc	tat	tat	gca	act	agt	ttg	gca	gat	ggg	gtc	cca	tca	240	
Lys	Leu	Leu	Ile	Tyr	Tyr	Ala	Thr	Ser	Leu	Ala	Asp	Gly	Val	Pro	Ser		
45						50						55			60		
agg	ttc	agt	ggc	tcc	gga	tct	ggg	acc	gat	tac	act	ctc	acc	atc	tcg	288	
Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Tyr	Thr	Leu	Thr	Ile	Ser		
			65						70						75		
agc	ctc	cag	cct	gaa	gat	ttt	gca	act	tac	tat	tgt	ctg	cag	cat	ggg	336	
Ser	Leu	Gln	Pro	Glu	Asp	Phe	Ala	Thr	Tyr	Tyr	Cys	Leu	Gln	His	Gly		
			80						85						90		
gag	agc	ccg	tac	acg	ttc	ggc	gga	ggg	acc	aag	gtg	gag	atc	aaa		381	
Glu	Ser	Pro	Tyr	Thr	Phe	Gly	Gly	Gly	Thr	Lys	Val	Glu	Ile	Lys			
95						100						105					

<210> 88

<211> 107

<212> PRT

<213> Artificial Sequence

 $\langle 220 \rangle$

<223> Amino acid sequence of version "b" of humanized L chain V region

<400> 88

Asp	Ile	Gln	Met	Thr	Gln	Ser	Pro	Ser	Ser	Leu	Ser	Ala	Ser	Val	Gly
1				5					10					15	
Asp	Arg	Val	Thr	Ile	Thr	Cys	Lys	Ala	Ser	Gln	Asp	Ile	Lys	Ser	Phe
			20					25					30		
Leu	Ser	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Lys	Ala	Pro	Lys	Leu	Leu	Ile
		35					40					45			
Tyr	Tyr	Ala	Thr	Ser	Leu	Ala	Asp	Gly	Val	Pro	Ser	Arg	Phe	Ser	Gly
	50					55					60				
Ser	Gly	Ser	Gly	Thr	Asp	Tyr	Thr	Leu	Thr	Ile	Ser	Ser	Leu	Gln	Pro
65					70					75					80
Glu	Asp	Phe	Ala	Thr	Tyr	Tyr	Cys	Leu	Gln	His	Gly	Glu	Ser	Pro	Tyr
				85					90					95	
Thr	Phe	Gly	Gly	Gly	Thr	Lys	Val	Glu	Ile	Lys					
			100					105							

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gag agc ccg tac acg ttc ggc gga ggg acc aag gtg gag atc aaa 381
 Glu Ser Pro Tyr Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
 95 100 105

<210> 90

<211> 107

<212> PRT

<213> Artificial Sequence

<220>

<223> Amino acid sequence of version "c" of humanized L
 chain V region

<400> 90

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
 1 5 10 15
 Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Ile Lys Ser Phe
 20 25 30
 Leu Ser Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
 35 40 45
 Tyr Tyr Ala Thr Ser Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
 50 55 60
 Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro
 65 70 75 80
 Glu Asp Ile Ala Thr Tyr Tyr Cys Leu Gln His Gly Glu Ser Pro Tyr
 85 90 95
 Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
 100 105

<210> 91

<211> 72

<212> DNA

<213> Artificial Sequence

<220>

<223> FR shuffling primer F2SS

<400> 91

gtctcttaag ttggttccag cagaaaccag ggaaatctcc taagaccctg atctactatg 60
 caactagtaa ca 72

<210> 92

<211> 72

<212> DNA
 <213> Artificial Sequence
 <220>
 <223> FR shuffling primer F2SA
 <400> 92
 tggtactagt tgcataatag atcagggact taggggcttt ctctggtttc tgctgatacc 60
 aacttaagag ac 72
 <210> 93
 <211> 72
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> FR shuffling primer F2XS
 <400> 93
 gtctcttaag ttggtatcag cagaaaccag agaaagcccc taagtccttg atctattatg 60
 caactagtaa ca 72
 <210> 94
 <211> 72
 <212> DNA
 <213> Artificial Sequence
 <220>
 <223> FR shuffling primer F2XA
 <400> 94
 tggtactagt tgcataatag atcagggact taggggcttt ctctggtttc tgctgatacc 60
 aacttaagag ac 72
 <210> 95
 <211> 381
 <212> DNA
 <213> Artificial Sequence
 <220>
 <221> sig-peptide
 <222> (1)...(60)
 <220>
 <221> mat-peptide
 <222> (61)...(381)

<400> 96

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
 1 5 10 15
 Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Ile Lys Ser Phe
 20 25 30
 Leu Ser Trp Phe Gln Gln Lys Pro Gly Lys Ser Pro Lys Thr Leu Ile
 35 40 45
 Tyr Tyr Ala Thr Ser Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly
 50 55 60
 Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro
 65 70 75 80
 Glu Asp Phe Ala Thr Tyr Tyr Cys Leu Gln His Gly Glu Ser Pro Tyr
 85 90 95
 Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
 100 105

<210> 97

<211> 381

<212> DNA

<213> Artificial Sequence

<220>

<221> sig-peptide

<222> (1)...(60)

<220>

<221> mat-peptide

<222> (61)...(381)

<223> Nucleotide sequence coding for version "b2" of
 humanized L chain V region

<400> 97

atg agg gcc cct gct cag ttt ttt ggg atc ttg ttg ctc tgg ttt cca 48
 Met Arg Ala Pro Ala Gln Phe Phe Gly Ile Leu Leu Leu Trp Phe Pro
 -20 -15 -10 -5
 ggg atc cga tgt gac atc cag atg acc cag tct cca tcc tcc ctg tct 96
 Gly Ile Arg Cys Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser
 1 5 10
 gca tct gta gga gac aga gtc acc atc acg tgc aag gcc tca cag gac 144
 Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp
 15 20 25

att	aaa	agc	ttc	tta	agt	tgg	tat	cag	cag	aaa	cca	gag	aaa	gcc	cct	192	
Ile	Lys	Ser	Phe	Leu	Ser	Trp	Tyr	Gln	Gln	Lys	Pro	Glu	Lys	Ala	Pro		
30						35						40					
aag	tcc	ctg	atc	tat	tat	gca	act	agt	ttg	gca	gat	ggg	gtc	cca	tca	240	
Lys	Ser	Leu	Ile	Tyr	Tyr	Ala	Thr	Ser	Leu	Ala	Asp	Gly	Val	Pro	Ser		
45						50						55			60		
agg	ttc	agt	ggc	tcc	gga	tct	ggg	acc	gat	tac	act	ctc	acc	atc	tcg	288	
Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Tyr	Thr	Leu	Thr	Ile	Ser		
			65						70						75		
agc	ctc	cag	cct	gaa	gat	ttt	gca	act	tac	tat	tgt	ctg	cag	cat	ggg	336	
Ser	Leu	Gln	Pro	Glu	Asp	Phe	Ala	Thr	Tyr	Tyr	Cys	Leu	Gln	His	Gly		
			80						85						90		
gag	agc	ccg	tac	acg	ttc	ggc	gga	ggg	acc	aag	gtg	gag	atc	aaa		381	
Glu	Ser	Pro	Tyr	Thr	Phe	Gly	Gly	Gly	Thr	Lys	Val	Glu	Ile	Lys			
95						100						105					

<210> 98

<211> 107

<212> PRT

<213> Artificial Sequence

 $\langle 220 \rangle$

<223> Amino acid sequence of version "b2" of humanized L chain V region

<400> 98

Asp	Ile	Gln	Met	Thr	Gln	Ser	Pro	Ser	Ser	Leu	Ser	Ala	Ser	Val	Gly
1				5					10					15	
Asp	Arg	Val	Thr	Ile	Thr	Cys	Lys	Ala	Ser	Gln	Asp	Ile	Lys	Ser	Phe
			20					25					30		
Leu	Ser	Trp	Tyr	Gln	Gln	Lys	Pro	Glu	Lys	Ala	Pro	Lys	Ser	Leu	Ile
		35					40					45			
Tyr	Tyr	Ala	Thr	Ser	Leu	Ala	Asp	Gly	Val	Pro	Ser	Arg	Phe	Ser	Gly
	50					55					60				
Ser	Gly	Ser	Gly	Thr	Asp	Tyr	Thr	Leu	Thr	Ile	Ser	Ser	Leu	Gln	Pro
65					70					75					80
Glu	Asp	Phe	Ala	Thr	Tyr	Tyr	Cys	Leu	Gln	His	Gly	Glu	Ser	Pro	Tyr
				85					90					95	
Thr	Phe	Gly	Gly	Gly	Thr	Lys	Val	Glu	Ile	Lys					
			100					105							

<210> 99

<211> 117

<212> PRT

<213> Mouse

<220>

<223> Amino acid sequence of H chain V region of anti TF
mouse monoclonal antibody ATR-5

<400> 99

Glu Val Gln Leu Gln Gln Ser Gly Thr Asn Leu Val Arg Pro Gly Ala
5 10 15

Leu Val Lys Leu Ser Cys Lys Gly Ser Gly Phe Asn Ile Lys Asp Tyr
20 25 30

Tyr Met His Trp Val Lys Gln Arg Pro Glu Gln Gly Leu Glu Trp Ile
35 40 45

Gly Gly Asn Asp Pro Ala Asn Gly His Ser Met Tyr Asp Pro Lys Phe
50 55 60

Gln Gly Lys Ala Ser Ile Thr Ala Asp Thr Ser Ser Asn Thr Ala Tyr
65 70 75 80

Leu Gln Leu Ser Ser Leu Thr Ser Glu Asp Thr Ala Val Tyr Phe Cys
85 90 95

Ala Arg Asp Ser Gly Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Ser
100 105 110

Val Thr Val Ser Ser
115

<210> 100

<211> 107

<212> PRT

<213> Mouse

<220>

<223> Amino acid sequence of L chain V region of anti TF
mouse monoclonal antibody ATR-5

<400> 100

Asp Ile Lys Met Thr Gln Ser Pro Ser Ser Met Tyr Ala Ser Leu Gly
5 10 15

Glu Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Ile Lys Ser Phe
20 25 30

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<210> 101
<211> 780
<212> DNA
<213> Homosapiens
<220>
<223> DNA coding for soluble human TF
<400> 101
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atg	gag	acc	cct	gcc	tgg	ccc	cgg	gtc	ccg	cgc	ccc	gag	acc	gcc	gtc	48
Met	Glu	Thr	Pro	Ala	Trp	Pro	Arg	Val	Pro	Arg	Pro	Glu	Thr	Ala	Val	
		-30					-25					-20				
gct	cgg	acg	ctc	ctg	ctc	ggc	tgg	gtc	ttc	gcc	cag	gtg	gcc	ggc	gct	96
Ala	Arg	Thr	Leu	Leu	Leu	Gly	Trp	Val	Phe	Ala	Gln	Val	Ala	Gly	Ala	
		-15				-10					-5				-1	
tca	ggc	act	aca	aat	act	gtg	gca	gca	tat	aat	tta	act	tgg	aaa	tca	144
Ser	Gly	Thr	Thr	Asn	Thr	Val	Ala	Ala	Tyr	Asn	Leu	Thr	Trp	Lys	Ser	
	1			5					10					15		
act	aat	ttc	aag	aca	att	ttg	gag	tgg	gaa	ccc	aaa	ccc	gtc	aat	caa	192
Thr	Asn	Phe	Lys	Thr	Ile	Leu	Glu	Trp	Glu	Pro	Lys	Pro	Val	Asn	Gln	
			20					25					30			
gtc	tac	act	gtt	caa	ata	agc	act	aag	tca	gga	gat	tgg	aaa	agc	aaa	240
Val	Tyr	Thr	Val	Gln	Ile	Ser	Thr	Lys	Ser	Gly	Asp	Trp	Lys	Ser	Lys	
		35					40				45					
tgc	ttt	tac	aca	aca	gac	aca	gag	tgt	gac	ctc	acc	gac	gag	att	gtg	288
Cys	Phe	Tyr	Thr	Thr	Asp	Thr	Glu	Cys	Asp	Leu	Thr	Asp	Glu	Ile	Val	
	50					55				60						

53/57

<223> Amino acid sequence of soluble human TF

<400> 102

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Met Glu Thr Pro Ala Trp Pro Arg Val Pro Arg Pro Glu Thr Ala Val
      -30              -25              -20

Ala Arg Thr Leu Leu Leu Gly Trp Val Phe Ala Gln Val Ala Gly Ala
      -15              -10              -5              -1

Ser Gly Thr Thr Asn Thr Val Ala Ala Tyr Asn Leu Thr Trp Lys Ser
  1              5              10              15

Thr Asn Phe Lys Thr Ile Leu Glu Trp Glu Pro Lys Pro Val Asn Gln
      20              25              30

Val Tyr Thr Val Gln Ile Ser Thr Lys Ser Gly Asp Trp Lys Ser Lys
      35              40              45

Cys Phe Tyr Thr Thr Asp Thr Glu Cys Asp Leu Thr Asp Glu Ile Val
      50              55              60

Lys Asp Val Lys Gln Thr Tyr Leu Ala Arg Val Phe Ser Tyr Pro Ala
      65              70              75              80

Gly Asn Val Glu Ser Thr Gly Ser Ala Gly Glu Pro Leu Tyr Glu Asn
      85              90              95

Ser Pro Glu Phe Thr Pro Tyr Leu Glu Thr Asn Leu Gly Gln Pro Thr
      100             105             110

Ile Gln Ser Phe Glu Gln Val Gly Thr Lys Val Asn Val Thr Val Glu
      115             120             125

Asp Glu Arg Thr Leu Val Arg Arg Asn Asn Thr Phe Leu Ser Leu Arg
      130             135             140

Asp Val Phe Gly Lys Asp Leu Ile Tyr Thr Leu Tyr Tyr Trp Lys Ser
      145             150             155             160

Ser Ser Ser Gly Lys Lys Thr Ala Lys Thr Asn Thr Asn Glu Phe Leu
      165             170             175

Ile Asp Val Asp Lys Gly Glu Asn Tyr Cys Phe Ser Val Gln Ala Val
      180             185             190

Ile Pro Ser Arg Thr Val Asn Arg Lys Ser Thr Asp Ser Pro Val Glu
      195             200             205

Cys Met Gly Gln Glu Lys Gly Glu Phe Arg Glu Asp Tyr Lys Asp Asp
      210             215             220

Asp Asp Lys
225
<210> 103

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<211> 780

<212> DNA

<213> Homosapiens

<220>

<223> DNA coding for human TF

<400> 103

atg gag acc cct gcc tgg ccc cgg gtc ccg cgc ccc gag acc gcc gtc	48
MET Glu Thr Pro Ala Trp Pro Arg Val Pro Arg Pro Glu Thr Ala Val	
-30 -25 -20	
gct cgg acg ctc ctg ctc ggc tgg gtc ttc gcc cag gtg gcc ggc gct	96
Ala Arg Thr Leu Leu Leu Gly Trp Val Phe Ala Gln Val Ala Gly Ala	
-15 -10 -5 -1	
tca ggc act aca aat act gtg gca gca tat aat tta act tgg aaa tca	144
Ser Gly Thr Thr Asn Thr Val Ala Ala Tyr Asn Leu Thr Trp Lys Ser	
1 5 10 15	
act aat ttc aag aca att ttg gag tgg gaa ccc aaa ccc gtc aat caa	192
Thr Asn Phe Lys Thr Ile Leu Glu Trp Glu Pro Lys Pro Val Asn Gln	
20 25 30	
gtc tac act gtt caa ata agc act aag tca gga gat tgg aaa agc aaa	240
Val Tyr Thr Val Gln Ile Ser Thr Lys Ser Gly Asp Trp Lys Ser Lys	
35 40 45	
tgc ttt tac aca aca gac aca gag tgt gac ctc acc gac gag att gtg	288
Cys Phe Tyr Thr Thr Asp Thr Glu Cys Asp Leu Thr Asp Glu Ile Val	
50 55 60	
aag gat gtg aag cag acg tac ttg gca cgg gtc ttc tcc tac ccg gca	336
Lys Asp Val Lys Gln Thr Tyr Leu Ala Arg Val Phe Ser Tyr Pro Ala	
65 70 75 80	
ggg aat gtg gag agc acc ggt tct gct ggg gag cct ctg tat gag aac	384
Gly Asn Val Glu Ser Thr Gly Ser Ala Gly Glu Pro Leu Tyr Glu Asn	
85 90 95	
tcc cca gag ttc aca cct tac ctg gag aca aac ctc gga cag cca aca	432
Ser Pro Glu Phe Thr Pro Tyr Leu Glu Thr Asn Leu Gly Gln Pro Thr	
100 105 110	
att cag agt ttt gaa cag gtg gga aca aaa gtg aat gtg acc gta gaa	480
Ile Gln Ser Phe Glu Gln Val Gly Thr Lys Val Asn Val Thr Val Glu	
115 120 125	

-1

Ser Gly Thr Thr Asn Thr Val Ala Ala Tyr Asn Leu Thr Trp Lys Ser
 1 5 10 15
 Thr Asn Phe Lys Thr Ile Leu Glu Trp Glu Pro Lys Pro Val Asn Gln
 20 25 30
 Val Tyr Thr Val Gln Ile Ser Thr Lys Ser Gly Asp Trp Lys Ser Lys
 35 40 45
 Cys Phe Tyr Thr Thr Asp Thr Glu Cys Asp Leu Thr Asp Glu Ile Val
 50 55 60
 Lys Asp Val Lys Gln Thr Tyr Leu Ala Arg Val Phe Ser Tyr Pro Ala
 65 70 75 80
 Gly Asn Val Glu Ser Thr Gly Ser Ala Gly Glu Pro Leu Tyr Glu Asn
 85 90 95
 Ser Pro Glu Phe Thr Pro Tyr Leu Glu Thr Asn Leu Gly Gln Pro Thr
 100 105 110
 Ile Gln Ser Phe Glu Gln Val Gly Thr Lys Val Asn Val Thr Val Glu
 115 120 125
 Asp Glu Arg Thr Leu Val Arg Arg Asn Asn Thr Phe Leu Ser Leu Arg
 130 135 140
 Asp Val Phe Gly Lys Asp Leu Ile Tyr Thr Leu Tyr Tyr Trp Lys Ser
 145 150 155 160
 Ser Ser Ser Gly Lys Lys Thr Ala Lys Thr Asn Thr Asn Glu Phe Leu
 165 170 175
 Ile Asp Val Asp Lys Gly Glu Asn Tyr Cys Phe Ser Val Gln Ala Val
 180 185 190
 Ile Pro Ser Arg Thr Val Asn Arg Lys Ser Thr Asp Ser Pro Val Glu
 195 200 205
 Cys MET Gly Gln Glu Lys Gly Glu Phe Arg Glu Ile Phe Tyr Ile Ile
 210 215 220
 Gly Ala Val Val Phe Val Val Ile Ile Leu Val Ile Ile Leu Ala Ile
 225 230 235 240
 Ser Leu His Lys Cys Arg Lys Ala Gly Val Gly Gln Ser Trp Lys Glu
 245 250 255
 Asn Ser Pro Leu Asn Val Ser
 260

DEPOSITOR

Name: CHUGAI SEIYAKU
KABUSHIKI KAISHA

Representative: Osamu Nagayama

Address: 5-1, Ukima 5-chome
Kita-ku, Tokyo

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF
MICROORGANISMS FOR THE PURPOSES OF
PATENT PROCEDURE

RECEIPT IN THE CASE OF AN ORIGINAL
DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page.

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the (Deposition Number)
DEPOSITOR: KPMM2
FERM BP-7419

II. SCIENTIFIC DESCRIPTION AND/OR TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

- x a scientific description
- x a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the
microorganism identified under I above, which was received by it on
February 22, 1994 (Date of the original deposit)¹.

IV. RECEIPT OF TRANSFER

This International Depositary Authority accepted the
microorganisms identified under I above, on February 22, 1994 (Date of the
original deposit), and accepted a request for transfer to a deposition
under Budapest treaty from the original deposition, on December 27, 2000.
(Transferred from FERM P-14170 deposited on February 22, 1994.)

V. INTERNATIONAL DEPOSITARY AUTHORITY

National Institute of Science and Human-Technology

Agency of Industrial Science and Technology

Director General Shinichi Ohashi

1-3, Higashi 1 chome, Tsukuba-shi,
Ibaraki-ken, 305-8566, Japan

December 27, 2000

国際様式

INTERNATIONAL FORM

10080501.042202

特許手続上の微生物の寄託の国際的承認
に関するブタペスト条約

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF
MICROORGANISMS FOR THE PURPOSES OF
PATENT PROCEDURE

RECEIPT IN THE CASE OF AN ORIGINAL
DEPOSIT

下記国際寄託当局によって規則 7. 1 に従い
発行される。

issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this
page.

原寄託についての受託証

氏名 (名称)

中外製薬株式会社
取締役社長 永山 治

寄託者

あ て 名 氏

東京都北区浮間 5 丁目 5 番 1 号

殿

1. 微生物の表示

(寄託者が付した識別のための表示)
KPMM2

(受託番号)
FERM BP- 7419

2. 科学的性質及び分類学上の位置

1 欄の微生物には、次の事項を記載した文書が添付されていた。

- 科学的性質
- 分類学上の位置

3. 受領及び受託

本国際寄託当局は、平成 6 年 2 月 22 日 (原寄託日) に受領した 1 欄の微生物を受託する。

4. 移管請求の受領

本国際寄託当局は、平成 6 年 2 月 22 日 (原寄託日) に 1 欄の微生物を受領した。
そして、平成 12 年 12 月 27 日 に原寄託よりブタペスト条約に基づく寄託への移管請求を受領した。
(平成 6 年 2 月 22 日 に寄託された微工研菌寄第 P- 14170 号より移管)

5. 国際寄託当局

通商産業省工業技術院生命工学工業技術研究所

名称: National Institute of Bioscience and Human-Technology
Agency of Industrial Science and Technology

所長 大箸 信

Dr. Shinichi Ohnishi Director-General

あて名: 日本国茨城県つくば市東 1 丁目 1 番 3 号 (郵便番号 305-8566)
1-3, Higashi 1 chome Tsukuba-shi Ibaraki-ken
305-8566, JAPAN

平成 12 年 (2000) 12 月 27 日